CHANGES IN EXPRESSION OF A MAJOR SIALOGLYCOPROTEIN ASSOCIATED WITH ASCITES FORMS OF A MAMMARY ADENOCARCINOMA

By

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NOMENCLATURE

ASGP-1	- major sialoglycoprotein of 13762 ascites tumors
Con A	- Concanavalin A
cpm	- counts per minute
da	- day(s)
DMSO	- dimethyl sulfoxide
DPBS	- Dulbecco's phosphate buffered saline
dpm	- disintegrations per minute
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethyleneglycol-bis-(β-amino-ethyl ether)N, N'-tetraacetic acid
F344	- Fisher 344 rats; only female rats used in study
FITC-Con A	- fluorescein conjugated Concanavalin A
g	- a unit of acceleration equal to the acceleration of gravity
Gal	- galactose
GalNAc	- N-acetylgalactosamine
Gdn HCl	- guanidine hydrochloride
M	- blood-group M antigen
MAT	- prefix used to indicate ascites forms of 13762 tumor
mCi	- millicurie
MEM	- minimal essential media
MR	- cell culture forms of the 13762 adenocarcinoma

cMR6, cMRX	- X=8, 9, 10 Cloned variants of the 13762 cell culture tumor line
N	- blood-group N antigen
NA-Gal	- N-acetylgalactosamine
NAG	- N-acetylglucosamine
PBS	- phosphate buffered saline
PNA	- peanut agglutinin
PPO	- 2,5-diphenyloxazole
SBF	- specific blocking factor
SDS	- sodium dodecyl sulfate
SEM	- scanning electron microscopy
T-antigen	- Thomsen Friedenrich antigen
TA3-Ha	- Epiglycanin containing TA3 mammary carcinoma subline
TA3-St	- TA3 mammary carcinoma subline lacking glycoprotein Epiglycanin
TCA	- trichloracetic acid
TRIS	- tris-(hydroxymethyl)-aminomethane
TSTA	- tumor-specific transplantation antigen

CHAPTER I

INTRODUCTION

The Role of Glycoproteins in Oncogenesis

In order to understand the importance of glycoproteins in neoplastic disease, one must first be cognizant of their normal functions. Thus, it is advantageous to consider the general role of cell surface glycoproteins before discussing glycoproteins and cancer or the importance of glycoproteins in tumor immunology. One must also consider other facets of cancer development, including abnormal membrane turnover and metastasis. Careful analysis of specific tumor systems such as the TA3 mammary carcinoma and the 13762 rat mammary adenocarcinoma enables the individual to gain a clearer understanding of the complex, often deadly, diseases known as cancer.

Cell Surface Glycoproteins

Cell surface glycoproteins have been implicated in many important biological processes, including cell recognition and adhesion (Edwards, 1978), contact inhibition (Knecht, 1977), embryological development (Mintz and Glaser, 1978; Parish and Schmidlin, 1979) and membrane transport (Olden et al., 1979; reviews: Hughes, 1976; Glick and Flowers, 1978). The plasma membrane is enriched in this class of proteins, and glycoproteins have a definite topographical organization in this membrane.

The carbohydrate residues are generally confined to that end of the protein which is exposed on the external surface of the lipid bilayer (Marchesi et al., 1972; Hirano et al., 1972; Singer, 1974).

Current evidence indicates that the oligosaccharide portion of glycoproteins is important for protein secretion (Eylar, 1965), specific recognition of serum glycoproteins (Lunney and Ashwell, 1976), protection of glycoproteins against proteolytic degradation (Olden, Pratt and Yamada, 1978), the insertion or proper orientation of glycoproteins in the plasma membrane (Pouyssegur and Pastan, 1976; Hughes et al., 1977), and for cell adhesion and determining cell morphology (Duksin and Bornstein, 1977). Despite this importance, glycoprotein structure-function relationships are not well understood.

Glycoproteins and Cancer

During the last decade, many investigators have found changes in cell surface glycoproteins associated with oncogenesis. Cell surface glycoprotein differences between normal and malignant cells in culture have been demonstrated by analysis of chromatographic patterns of glycoproteins released from the cell surfaces with trypsin and degraded with Pronase (Buck, Glick and Warren, 1971).

Interest in the relationship of glycoproteins to cancer arose out of the observation that patients with neoplastic disease had elevated plasma levels of protein-bound carbohydrate (Winkler and Bekesi, 1970). Subsequent investigations in many different laboratories have generally substantiated this finding, and there are some indications that there may be very specific glycoprotein changes characteristic of some types of malignant tumors (Bramwell and Harris, 1978). Antigens common to

cell surfaces of several human melanomas have been detected (Bystryn and Smalley, 1977), and a common and identical antigen has been isolated from two distinct human lung tumor extracts (Gaffer et al., 1979).

Furthermore, certain plant lectins which bind specifically to carbohydrates will cause agglutination of many malignant cells at a much lower lectin concentration than required to agglutinate their non-malignant counterparts (Burger, 1973). Agglutination, however, is a complex process which involves more than the number of lectin binding sites on a cell surface (reviews: Nicolson, 1974; Kornfeld and Kornfeld, 1978).

Peanut lectin, specific for terminal D-galactosyl residues (D-Gal-β(1→3)-GalNAc), is especially noteworthy. This oligosaccharide structure is found on the Thomsen-Friedenrich antigen (T-antigen) which is a precursor of the M and N blood-group antigens. According to present knowledge, T-antigen does not occur in unmasked form in healthy human tissues. However, this precursor T-antigen has been demonstrable in malignant tissue, but not in benign lesions or normal tissue, from human mammary glands. T-antigen has also been found in the epithelial layer of human stomach carcinoma and two human colon carcinoma-derived tissue culture lines (reviewed by Springer, 1977). Freeman and Kim (Cancer Bulletin) found that anti-T antibody level is severely depressed in patients with malignant breast and gastrointestinal epithelial tissues. However, they were unable to distinguish between decreased antibody synthesis and continuous preferential in vivo absorption of anti-T by the T-antigens in malignant tissues.

The possible role of glycosyltransferases, in particular cell surface glycosyltransferases, in neoplastic phenomena has been a source of

considerable controversy. Warren et al. (1973) attributed the glycopeptide differences that they observed between normal and malignant cells in culture to differences in a specific sialyltransferase activity between the cell lines, while Bosmann and Hilf (1974) have shown an elevated serum sialyltransferase activity in a transplantable mammary tumor.

Bauer et al. (1977b) have found that specific alterations of fucose metabolism are a characteristic feature of Morris hepatomas and have suggested that the α-3-fucosyltransferase activity of human serum can be used as an indicator of malignancy (Bauer et al., 1977a). Bauer et al. (1978) have also shown a decrease of human serum fucosyltransferase associated with successful tumor therapy. Furthermore, Chatterjee and Kim (1978) have established a link between a 6-7 fold increase in a fucosyltransferase level and metastasis of six metastasizing rat mammary tumors compared to four nonmetastasizing strains. Whether or not such correlations are true of malignancy in general remains to be seen.

One might expect enhanced degradation of glycoproteins at the surface of tumors to account for their lack of cohesiveness and apparent weakened cell-cell interactions. Acceleration of these processes could be resonsible for the initial step in metastasis, through the release of some cancer cells from the primary tumor. Kim et al. (1975) have shown a correlation between metastasizing ability and absence of glycoprotein coat for a series of mammary tumors. Such changes at the cell surface may be related to the immunogenicity of the tumors. It has been suggested that proteolysis is important for growth inhibition due to high cell densities. Some tumors and transformed cells elicit a fibrinogen

activator (Unkeless et al., 1974), but this may not be related to growth inhibition (Mott et al., 1974). Changes in cell surface glycoproteins in transformed cells might be explained by action of proteases (Hynes, 1974). Likewise the glycoprotein differences described by Shin et al. (1975) in the R3230 Ac mammary tumor could have arisen from surface proteolysis.

Glycoproteins and Tumor Immunology

Because glycoproteins are major components of tumor (and normal) cell surfaces, it is logical to expect that they may play a role in the immune response of the host to the tumor. They may act as antigens themselves or as "masking agents" which block access to transplantation antigens, as proposed by Codington et al. (1973) for the TA3 mammary carcinoma.

In fact, most experimentally induced animal tumors and many human neoplasms express tumor-associated-antigens (Oettgen et al., 1972). Some of these are tumor specific transplantation antigens (TSTA), defined by their ability to induce an immune reaction which will lead to the rejection of transplanted neoplastic cells by the appropriately immune host (Old and Boyse, 1964; Sjogren, 1965). Other antigens do not function as TSTA but may be demonstrated by sensitive in vitro techniques (Hellstrom and Hellstrom, 1969). Phase specific (embryonic) tumor antigens shared by neoplastic and normal embryonic cells are examples of antigens which belong primarily to the latter group (Alexander, 1972). Still other tumors may not have any detectable antigens not also present on the corresponding normal cells.

Direct evidence for the involvement of cell surface carbohydrate

in the immune response is available from studies on the L1210 mouse leukemia (Bekesi et al., 1972). This tumor is readily transplantable under normal conditions, but is rejected if treated with neuraminidase before implantation. Neuraminidase treated cells are highly immunogenic and can be used for immunization of the murine host against subsequent tumor transplants. This phenomenon is not unique to cancer cells. The early mouse trophoblast may owe its privileged immunological position to a sialomucoprotein layer coating the trophoblastic epithelium (Currie et al., 1968). Neuraminidase treatment of ectoplacental cone cells "unmasks" the presence of transplantation antigens and reveals that there is no intrinsic deficit of such antigens in early mouse trophoblast. As mentioned previously, Kim et al. (1975) have suggested that metastasizing tumors have lost their glycocalyx, which was postulated to act as an antigen on the tumor cells. Isolated membranes and released glycoprotein materials were immunogenic in the rabbit and showed similar antigens. However, this does not establish their immunogenicity in the host.

The shedding of tumor antigens in general has been described by many authors (Rittenhouse et al., 1978; Lopez and Thomsen, 1977; Raz et al., 1978; Bystryn, 1977). One should consider what role they play in permitting the survival of the tumor from destruction by the immune system. One possibility is that the loss of cell surface antigens renders the cell unrecognizable as a foreign entity. A more likely mechanism, however, is that the materials "shed" from the cell surface combine with essential components of the immune system to render them inoperative. Thus, specific blocking factors (SBF) are defined as any humoral factors which can, in a specific way, impair an immune response to tumor antigens,

independent of their site of action and their molecular composition. SBF may be tumor products, antigen-antibody complexes or T-cell derived suppressor molecules. The importance of SBF has been recently reviewed (Hellstrom et al., 1977). There have been reports from several laboratories that SBF from mouse sarcomas inoculated into syngeneic animals can enhance the growth of tumor cells having the respective antigenic specificity (Pierce, 1971; Ran and Witz, 1972; Bansal et al., 1972). However, the nature of this blocking effect has not been completely deciphered.

Membrane Turnover

Warren (1969) proposed that surface membrane turnover might be an important aspect of the abnormal properties of tumors. Relatively little work has been done in the area of glycoprotein turnover and release from mammalian cells, because of the complexities of the membrane, the number of glycoproteins involved and the difficulties of isolating individual components for analysis. Schimke (1975) has reviewed the important aspects of protein turnover in animal cells, including methodology. Atkinson (1975) reported an investigation focused on turnover of fucosyl glycoproteins in animal cells. The results indicate that there is a significant internal pool of fucose-containing glycoprotein which is assembled into the membrane independently of nonfucosylated membrane proteins. Based on their research using hepatoma tissue culture cells, Doyle et al. (1978) have proposed that, during biogenesis, fucose-containing glycoproteins of the cell are assembled as a membrane-limited structure in the intracellular compartment. Part of this presynthesized membrane is then supplied directly to the plasma membrane,

but part of it is added to a relatively large internal pool of membrane also having the same glycoprotein composition as the plasma membrane. Dual label experiments utilizing a neuroblastoma cell line have shown that numerous surface glycoproteins are metabolized more rapidly or slowly than most of the cell surface constituents (Hudson and Johnson, 1977). Glycoproteins turning over more rapidly than other membrane components have also been observed in other cell systems (Goldberg, 1974).

Metastasis

Tumor metastasis is clinically one of the most grave aspects of cancer. The spread of tumor cells from the primary site to secondary sites occurs through the circulatory system, lymphatics or coelomic cavities (Coman, 1953; Zeidman, 1957). Free tumor cells or their aggregates in the blood will lodge in the first capillary bed they encounter, but may then re-enter the circulation and stop in other organs (Fisher and Fisher, 1967). Location of secondaries is dependent on the particular tumor type and is not necessarily a random process. Such behavior indicates that discrete tumor cell properties are responsible for the metastasizing ability of tumors. The importance of tissue dissociation and cell-cell interactions in the overall metastatic process indicates that the cell surface properties may be critical to metastasis. This hypothesis is supported by the work of Nicolson and Winkelhake (1975) on the relationship of the organ specificity of blood-born tumor metastases to cell adhesion and by the previously mentioned studies of Kim *et al.* (1975) on the correlation between metastasizing capability and glycocalyx. Cell surface phenomena clearly must be

involved in both the dissociation of the tumor cell from its primary site and its association with a new site.

However, neither invasion nor metastasis is peculiar to cancer; both phenomena are exemplified by the developing placenta and the spread to the liver and lungs of fragments of chorionic tissue which may implant and grow but which, with the cessation of the pregnancy, die and disappear. What is peculiar to cancer is the ability of the disseminated cells to continue to divide and thus increase in bulk (Davies, 1977).

TA3 Mammary Carcinoma

The TA3 mammary carcinoma sublines TA3-Ha and TA3-St have been studied extensively by Codington and coworkers. Epiglycanin is the sialoglycoprotein found in abundance on the TA3-Ha mammary adenocarcinoma, an allotransplantable subline which will also transplant across species barriers (Miller *et al.*, 1977). It is virtually absent from the strain-specific TA3-St subline, leading to the suggestion that the glycoprotein epiglycanin is the factor responsible for transplantability differences by virtue of its ability to block access to transplantation antigens (Sanford *et al.*, 1973). Epiglycanin content of cells or fluids can be assayed by its reaction with *Vicia graminea* lectin (Codington *et al.*, 1975). The glycoprotein is found in ascites fluids of the tumors, suggesting that it is shed from the cells (Cooper, Codington and Brown, 1974). The overall release of epiglycanin *in vitro* is biphasic (Miller and Cooper, 1978). When the TA3-Ha ascites cells were grown in suspension culture, epiglycanin gradually diminished such that after 7-10 days only 50% remained and after 2 months only 10% was detected.

Repassage of the cultured cells *in vivo* cuased the gradual reappearance of the glycoprotein (Miller *et al.*, 1975). Most of the work on the glycoprotein itself has been done on fragments released from the cells by proteolysis (Codington *et al.*, 1972). The fragments are large (5×10^5 daltons) and form long rod-like structures (Slayter and Codington, 1973). Composition studies show 70% carbohydrate and 30% protein with sialic acid, N-acetylglucosamine (NAG), N-acetylgalactosamine (NA-Gal) and galactose as the predominant sugars. Threonine and serine are the primary amino acids. In 6 M guanidine two components were found. The molecular weights were 88 K and 180 K by gel filtration and 138 K and 308 K by sedimentation equilibrium. Epiglycanin can be visualized by transmission electron microscopy as fibrils extending from the surfaces of microvilli of the TA3-Ha cells. It can also be visualized by staining with cationized ferritin (Miller, Hay and Codington, 1977). However, little is known about its mode of association with the membrane. The TA3-Ha cells tend to be poorly agglutinable with many lectins, including Con A, while the TA3-St cells are readily agglutinable (Friberg *et al.*, 1974).

Glycophorin

The most studied cell surface glycoprotein has been glycophorin, the major sialoglycoprotein of the human erythrocyte membrane (Marchesi *et al.*, 1972). It is an integral protein, firmly bound to the membrane, which requires detergent or organic solvent treatment to release it from the membrane (Marchesi and Andrews, 1971; Blumenfeld and Zvilichovsky, 1972). Its amino acid sequence has been determined and consists of a 64-residue N-terminal sequence that bears the carbohydrate, a 32-

residue internal hydrophobic sequence and a 35-residue C-terminal sequence (Tomita and Marchesi, 1975). It has been proposed that the molecule extends through the membrane with the internal sequence interacting with the lipid bilayer. The state of aggregation in the membrane is uncertain. Two predominant forms are seen on SDS-electrophoresis and appear to be a monomer and dimer in equilibrium (Marton and Garvin, 1973). The equilibrium can be shifted by changing the solubilization or electrophoresis conditions or by chemical modification of the protein (Silverberg, Chow and Marchesi, 1977). From both composition and sedimentation determinations the molecular weight is 31,000 of which approximately 55% is carbohydrate. Glycophorin is rich in sialic acid, galactose and NA-gal and has lesser amounts of NAG, fucose and mannose (Hudson et al., 1975). At least two types of oligosaccharide chains are present. One is a four unit "mucin-like" structure (Thomas and Winzler, 1969), while the other is a larger "plasma" type (Thomas and Winzler, 1971).

Previous Work on the 13762 Adenocarcinoma Sublines

The 13762 rat mammary adenocarcinoma is a dimethylbenzanthrene induced solid tumor (Segaloff, 1966). The tumor was adapted for ascites growth by Bogden at Mason Research Institute (Tumor Bank Inventory). He was able to isolate three pharmacologically distinct sublines: MAT-A, MAT-B and MAT-C. In addition, a cell culture line, MR, was derived from the MAT-B ascites line by growth in soft agar.

After a number of passages in Carraway's laboratory, the MAT-B and MAT-C ascites lines became the more stable MAT-B1 and MAT-C1 lines used

in this investigation. Histopathological examination confirms that these lines are still adenocarcinomas.

By scanning electron microscopy MAT-C1 cells exhibit a highly irregular surface, covered with microvilli extending as branched structures from the cell body. MAT-B1 cells have a more normal appearance, with unbranched microvilli, ruffles, ridges and blebs associated closely with the cell body (Sherblom et al., 1980c). Both MAT-B1 (nonxenotransplantable) and MAT-C1 (xenotransplantable) ascites sublines contain a major sialoglycoprotein (ASGP-1) with low electrophoretic mobility in dodecyl sulfate. ASGP-1 has been purified from membrane vesicles by extraction with 4 M guanidine hydrochloride. The composition of ASGP-1 from each subline is high in serine, threonine, galactosamine, glucosamine, galactose and sialic acid with carbohydrate compositions of 67% and 73% for MAT-B1 and MAT-C1, respectively. Amino acid compositions of ASGP-1 from the sublines are essentially the same. The major difference between them is a 3-fold greater content of sialic acid per unit protein for MAT-C1 ASGP-1 than MAT-B1 ASGP-1. Molecular weights of 570,000 (MAT-B1) and 690,000 (MAT-C1) were estimated by sedimentation velocity analysis and gel filtration in 4 M guanidine hydrochloride. Fractionation of oligosaccharides from alkaline-borohydride treated ASGP-1 on Biogel P4 gives patterns that are quantitatively different for MAT-B1 and MAT-C1 (Sherblom, Buck and Carraway, 1980a). ASGP-1 from MAT-B1 cells is also labeled by [^{35}S]sulfate, but MAT-C1 ASGP-1 is not. Treatment of the ascites cells with trypsin releases about 30% of the total cell sialic acid without significant decrease in cell viability (Buck, Sherblom and Carraway, 1979).

Although both sublines bind concanavalin A, the MAT-B1 cells are

readily agglutinated and show distinct receptor redistribution, whereas the MAT-C1 cells are not agglutinated and do not show long range receptor redistribution (Carraway et al., 1978).

It is thought that the composition of the ASGP-1 oligosaccharides may be an important factor determining the ability of the sublines to be transplanted across species histocompatability barriers (Sherblom Buck and Carraway, 1980a). However, other factors, such as the quantity and form of ASGP-1 shed in vivo or the rate of clearance of ASGP-1 from serum, may also be important.

CHAPTER II

CHANGES IN EXPRESSION OF A MAJOR SIALOGLYCOPROTEIN ASSOCIATED WITH ASCITES FORMS OF A MAMMARY ADENOCARCINOMA

Introduction

Ascites sublines of the 13762 rat mammary adenocarcinoma have a dominant, large sialoglycoprotein (ASGP-1) located on their cell surfaces (Carraway et al., 1978; Sherblom, Buck and Carraway, 1980a). ASGP-1 is not observed in the solid 13762 tumor from which the ascites sublines are derived (Carraway et al., 1978). These results suggest that the expression of ASGP-1 is related to the environment or growth conditions of the tumor. Somewhat similar results have been obtained with the TA3-Ha mouse mammary adenocarcinoma. Subcutaneous injection of the TA3-Ha ascites cells caused a solid tumor which had reduced amounts of a major sialoglycoprotein (Cooper et al., 1977). Additional passages as a solid tumor increased the level of this sialoglycoprotein to near that of the ascites form. When TA3-Ha cells were put into culture, their level of the sialoglycoprotein decreased substantially (Cooper et al., 1977; Miller et al., 1975).

Since cell surface sialoglycoproteins may be important to the survival of tumor cells, we have investigated changes in expression of

ASGP-1 in 13762 cells adapted for growth in cell culture (13762 MR). The subline in culture does not have detectable ASGP-1. Intraperitoneal transplantation produces ascites tumor cells which also do not have ASGP-1. However, with continued passage of the tumor in ascites form ASGP-1 is produced. No ASGP-1 was found in solid tumors obtained by subcutaneous injection of 13762 cells. Morphologically distinct clones isolated from the 13762 MR showed essentially the same behavior.

Materials

McCoy's 5A (modified), nonessentail amino acids, fetal calf serum, and Penicillin/Streptomycin solution were from Gibco, sodium pyruvate was from Microbiological Associates, lactoperoxidase, glucose oxidase and PPO (2,5-diphenyloxazole) were from Sigma, [^{3}H]$NaBH_4$ (specific activity greater than 100 mCi/mmole) was from New England Nuclear, D-1-[^{3}H]glucosamine HCl (2-6 Ci/mmole) and carrier-free [^{125}I]NaI were from Amersham/Searle. Soluene 350 and Instagel were from Packard, the Stadie-Riggs Tissue Slicer was from Thomas, and the concanavalin A and peanut lectins were from Miles-Yeda, Ltd.

Methods

Preparation of Cells

MAT-B1, a 13762 rat mammary gland ascites tumor subline (Carraway et al., 1978), was maintained by weekly intraperitoneal injection of approximately 2 x 10^6 cells in 0.25 ml of 0.9% NaCl into 60-90 day old female Fisher 344 rats. After 6 or 7 days the ascites cells were recovered from the peritoneal cavity by aspiration and washed three times by suspending in Dulbecco's phosphate-buffered saline, pH 7.4 (Dulbecco,

1954) or Hanks' phosphate-buffered saline (Hanks and Wallace, 1949) and centrifuging at 120 g for three minutes. Viability was determined by Trypan blue exclusion; only preparations of cells showing viability greater than 98% were used.

13762 solid mammary tumor was maintained in F344 female rats by monthly subcutaneous transfer of minced tumor sections using a sterile trocar. Tumor cells used in experiments were from three-week tumors (approximately 1 cm maximum length).

13762 MR cells, a cell culture variant of MAT-B cells obtained as a gift from Dr. Erling Jensen at the Human Cell Culture Bank of Mason Research Institute, Rockville, Md., were maintained in serial culture in milk dilution bottles in McCoy's 5A (modified) minimal essential medium (MEM) supplemented with 10% fetal calf serum, nonesential amino acids, 1 mM sodium pyruvate and penicillin/streptomycin. Stocks were passaged every seven days. Spinner cultures were started from milk dilution bottle stocks at a cell density 1-5 x 10^5 cells/ml three days before labeling or harvesting the cells. Cells were pelleted and resuspended in fresh media 24 hours before labeling or harvesting.

Production of Ascites Tumor from 13762 MR

Ascites tumors were produced from washed 13762 MR spinner culture cells suspended in 0.9% NaCl. 5 x 10^6 cells were injected into the peritoneal cavity of F344 female rats and grown approximately 14 days for the first few passages in the rats. Thereafter, 1.25 x 10^6 cells were injected every seven days.

Cell Labeling with Periodate-Borohydride

Labeling with periodate-borohydride was performed according to the

procedure of Gahmberg and Andersson (1977). Cells (1×10^7) were incubated for 10 minutes on ice in 1 ml of Hanks' PBS, pH 7.4, containing 2 mM sodium periodate; the reaction was quenched by addition of 0.2 ml of 0.1 M glycerol in PBS, and cells were washed once with Hanks' PBS, pH 8.0. The sample was suspended in 1 ml of the same buffer and incubated for 30 minutes at room temperature with 0.25 mCi $[^3H]NaBH_4$. Unlabeled $NaBH_4$ (0.1 mg) was added, and the cells were washed twice in the pH 8.0 buffer and once with PBS, pH 7.4.

Lactoperoxidase-Catalyzed Iodination

Lactoperoxidase-catalyzed iodination was performed according to the method of Hynes (1973). Carrier-free ^{125}I (0.1 mCi), lactoperoxidase (40 μg) and glucose oxidase (0.2 units) were added to a suspension of 1×10^7 cells in 1 ml of Hanks' PBS, pH 7.4, containing 1 mg/ml glucose. After incubation at room temperature for 10 minutes, cells were washed three times with PBS, pH 7.4 to which 1 mM KI was added.

$[^3H]$Glucosamine Labeling of Cells

Tritiated glucosamine (5 μCi/ml concentration) was diluted with 1 ml McCoy's 5A (modified) and added to the cell culture which was incubated with label in a 5% CO_2 atmosphere for 5-6 hours at 37^o before harvesting the cells. The cells were washed three times with Hanks' PBS, pH 7.4. 13762 solid tumor was similarily labeled after slicing into 0.5 mm thick slices using a Stadie-Riggs tissue unit.

Plasma Membrane Preparation

Plasma membranes were prepared by the Zn^{++} stabilization method

as reported previously (Carraway et al., 1976) except that 0.1% NaCl was added to the 40 mM Tris, pH 7.4, used in the swelling step and the incubation in $ZnCl_2$ was twelve minutes at room temperature.

Electrophoresis

Labeled whole cells or plasma membranes were solubilized by addition of an equal volume of solution containing 0.125 M Tris, pH 6.8, 3% SDS (sodium dodecyl sulfate) and 5% β-mercaptoethanol and immersion in a boiling water bath for five minutes. Solubilized whole cells were sonicated briefly at low frequency to disrupt viscous material, and 0.1 volume of 0.02% Pyronin Y in 50% glycerol was added.

The solubilized cells or plasma membranes were subjected to SDS polyacrylamide gel electrophoresis as described previously (Sherblom, Buck and Carraway, 1980a; King and Laemmli, 1971). After staining with Coomassie blue, gels were soaked 30 minutes in 10% methanol followed by 10% methanol, 1% glycerol. Gels to be processed for fluorography (those with tritiated samples) were soaked in three changes of DMSO (dimethyl sulfoxide) (20 minutes each) and 22% PPO in DMSO for 30 minutes (Bonner and Laskey, 1974). After a water wash the gels were dried, placed in contact with Kodak X-Omat R (XR-5) x-ray film which had been preflashed to increase sensitivity (Laskey and Mills, 1975) and stored at -70^{o} for 7-10 days, except where otherwise noted. Kodak no-screen film NS-ST was used for ^{125}I-autoradiography of slab gels.

Lectin "staining" of gels with ^{125}I-labeled lectins was performed as described previously (Sherblom, Buck and Carraway, 1980a; Burridge, 1976).

Cloning

A MR cell suspension was diluted to an average of 2 cells/ml and 5 cells/ml. 0.1 ml was then pipetted into each well of five Costar 96 well (6.4 mm diameter) tissue culture cluster plates. Medium used was 25% preconditioned refiltered McCoy's 5A (modified) plus 10% fetal calf serum. Seventeen hours after the dilution, each well was examined microscopically. Wells containing only a single cell were marked and periodically examined for colony growth. Cultures were maintained in a 5% CO_2 atmosphere until the third passage, when they were transferred to milk dilution bottles.

Electron Microscopy

Cells (10^7/ml) for scanning electron microscopy were added dropwise with shaking to ten volumes of 2% glutaraldehyde in 0.1 M sucrose, 0.1 M cacodylate, pH 7.4, at 37° and incubated for 120 minutes. The cells were then placed on serum-coated coverslips prepared by incubating sterile coverslips in 10% calf serum-MEM (Eagle's minimal essential medium) for at least two hours. The cells were allowed to settle onto the coverslips, which were left undisturbed at 4° overnight, washed twice in cacodylate-sucrose buffer and treated with 0.5% osmium tetroxide at room temperature for 60 minutes. The samples were dehydrated with a graded series of ethanol and dried at the critical point of CO_2 in a Polaron E3000 critical point drying apparatus. The coverslips were mounted on stubs and coated with 100 $\mathring{A}$ of gold with a Hummer II. Samples were viewed with a JEOL JSM-35 scanning electron microscope operated at 15 KV, and micrographs were recorded on Polaroid type 55 negative films.

Results

Description of the Cultured Subline

The 13762 MR cells were originally isolated by Dr. Erling Jensen from the MAT-B ascites form of the 13762 adenocarcinoma. Although the cells grow attached to a substratum, a considerable number of them release and float in the medium as the cell density increases. Time lapse cinematography shows that this release is not due to crowding and a failure to find sufficient room to spread after mitosis (Sandra McGuire, unpublished observations). Release appears to be a random event. If the attached and suspended populations are separated and recultured, the same proportion of attached and suspended cells are found when they reach the appropriate density. This behavior continued for at least 25 passages, indicating that there is no selection for either form.

The attached and suspended cells have similar morphologies by scanning electron microscopy (Figure 1). Moreover, cell surface and glycoprotein labeling experiments demonstrated no substantial differences in cell surface components between attached and suspended cells after electrophoresis (Figure 2). Likewise the polypeptides observed by Coomassie blue staining were essentially indistinguishable (Figure 3, gels C and D).

To show that the labeling procedures labeled primarily cell surface components, plasma membranes were isolated using the Zn^{++}-stabilization method (Carraway et al., 1976). Figure 4 shows that the labeling patterns, observed after polyacrylamide gel electrophoresis in dodecyl sulfate, are essentially the same for labeled cells and for membranes isolated from labeled cells. As expected the polypeptide patterns for cells and

Figure 1. Scanning Electron Micrographs of Attached (A) and Suspended (B) 13762 MR Cells. 5400 x magnification.

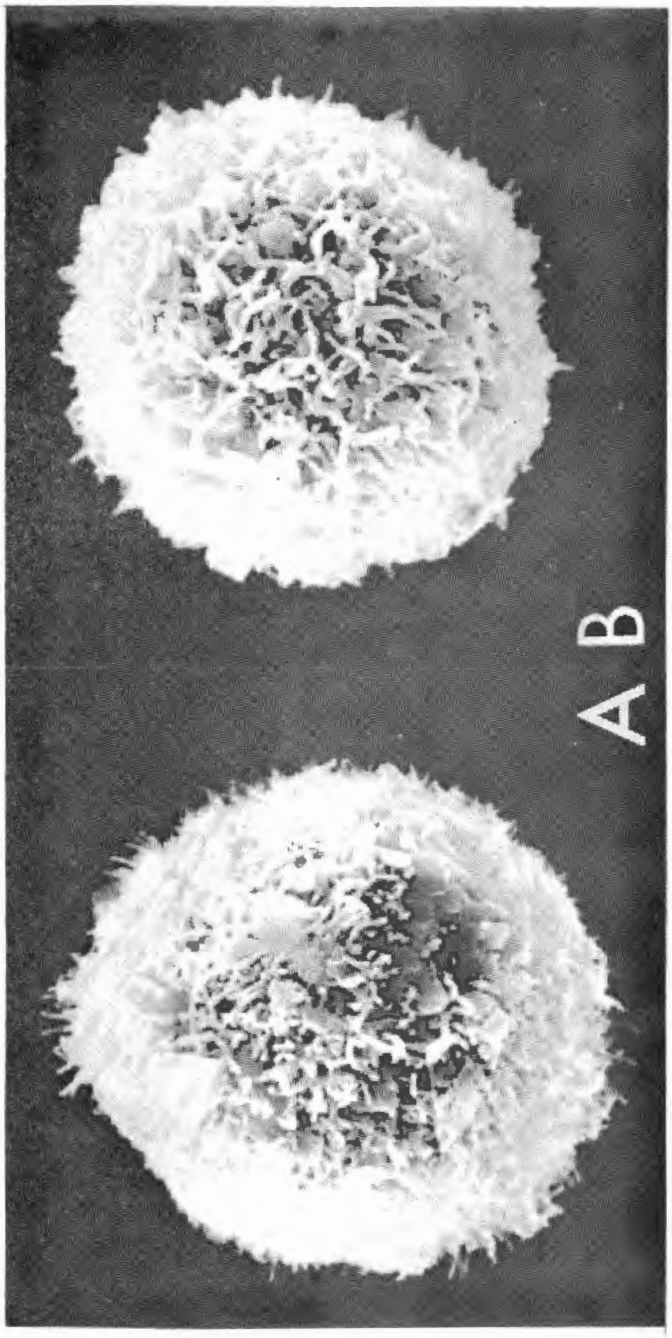

A
B

Figure 2. Comparison of SDS-Polyacrylamide (7.5%) Gel Profiles of MR Spinner Cells (A) to MR Cells Attached to Substratum (B). Cells are labeled by lactoperoxidase-catalyzed ^{125}I-iodination (Panel I), periodate [^{3}H]borohydride oxidation-reduction (Panel II), and [^{3}H]glucosamine metabolic labeling. The proteins used as molecular weight markers were myosin (210 K), phosphorylase (93 K), bovine serum albumin (68 K) and ovalbumin (43 K).

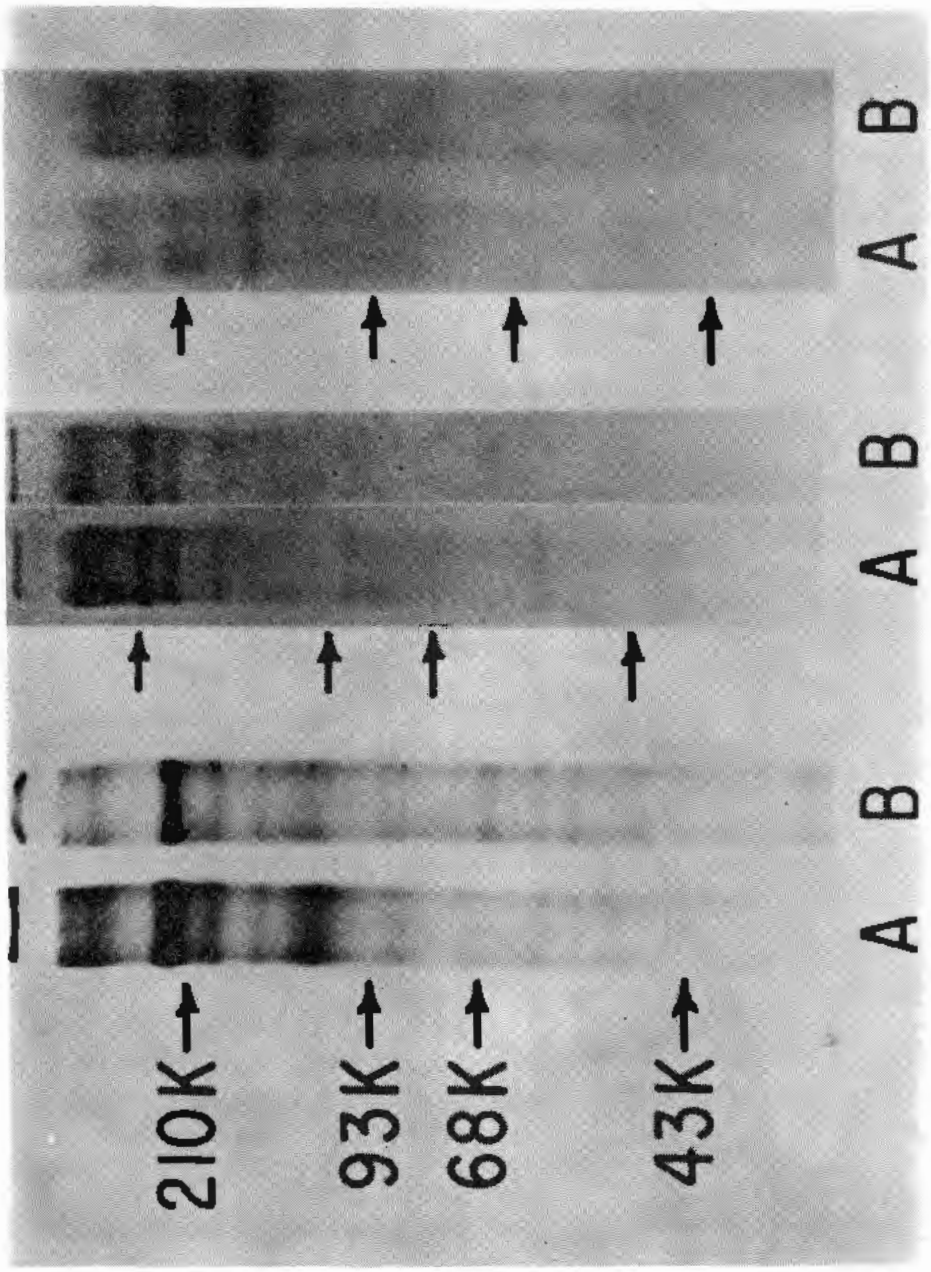

A B
A B
A B
210K
93K
68K
43K

Figure 3. Polypeptides Determined by SDS-Polyacrylamide (7.5%) Gel Electrophoresis of 13762 Solid Tumor (A), MAT-B1 Cells (B), MR Attached Cells (C), MR Spinner Cells (D) and MAT-MR1-S Ascites Cells (E).

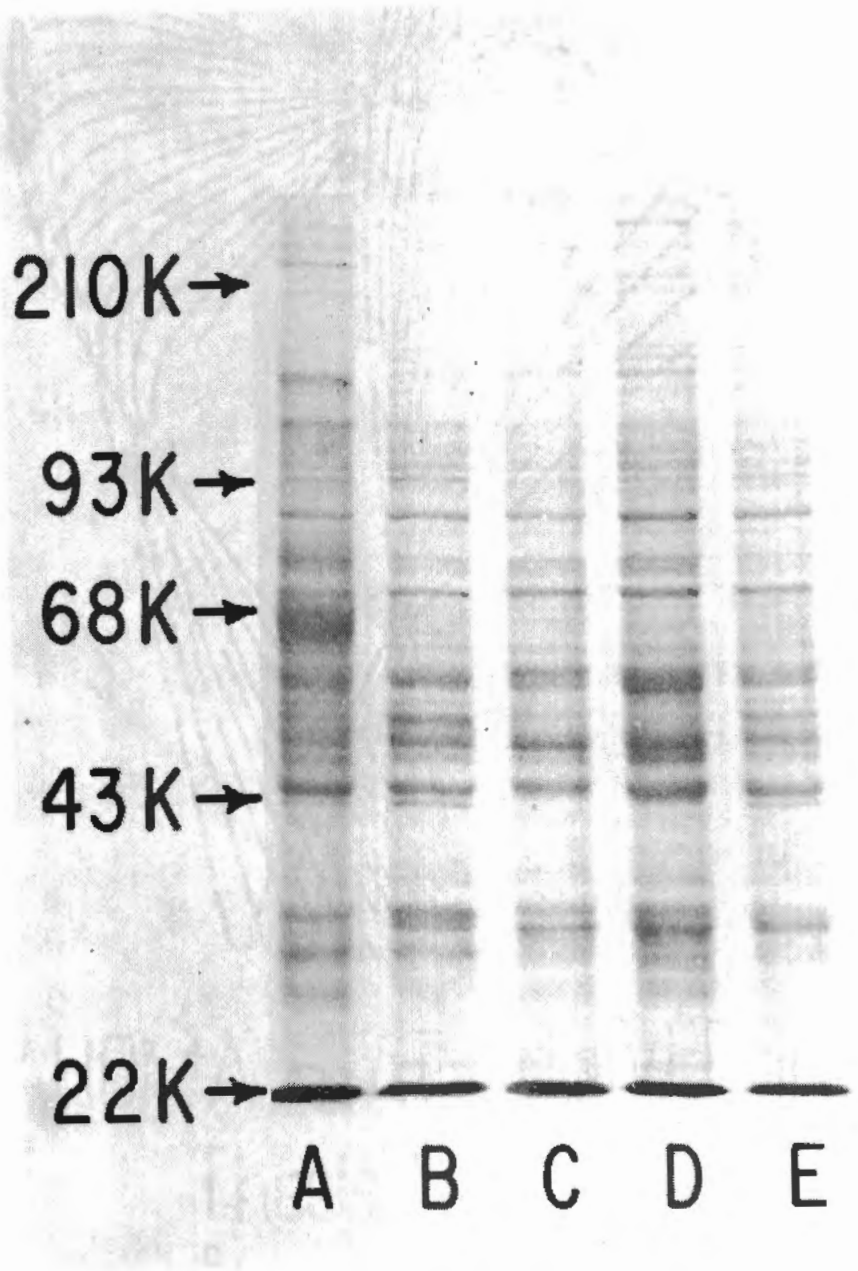
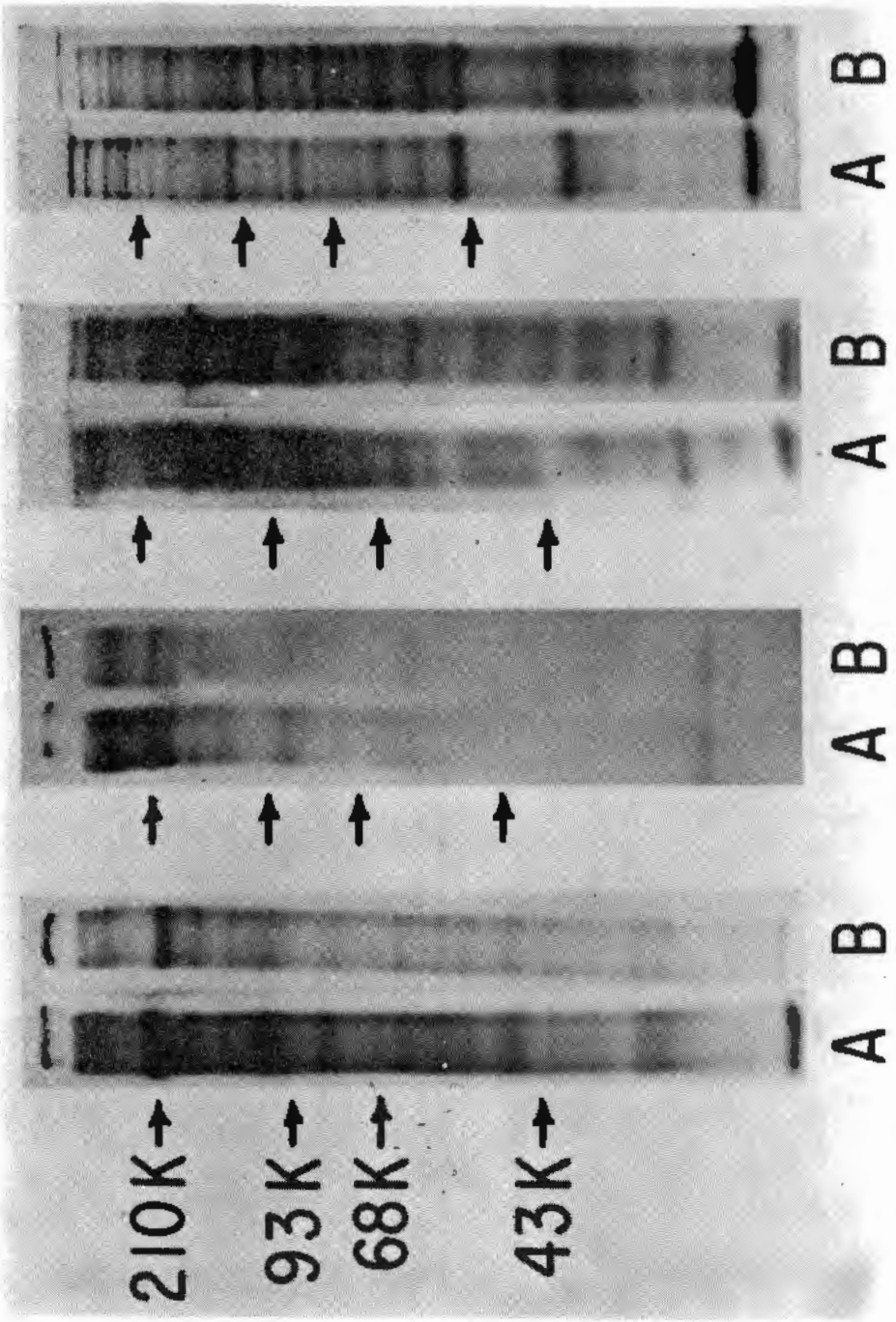

210K
93K
68K
43K
22K
A
B
C
D
E

Figure 4. Comparison of Labeling, Lectin and Polypeptide Profiles of Membranes Isolated form Labeled MR Spinner Culture Cells (A) to the Original Cells (B). Labeling was by lactoperoxidase catalyzed ^{125}I-iodination (Panel I), periodate-[^{3}H]borohydride oxidation-reduction (Panel II), [^{125}I]concanavalin A staining (Panel III), and coomassie-blue staining (Panel IV).

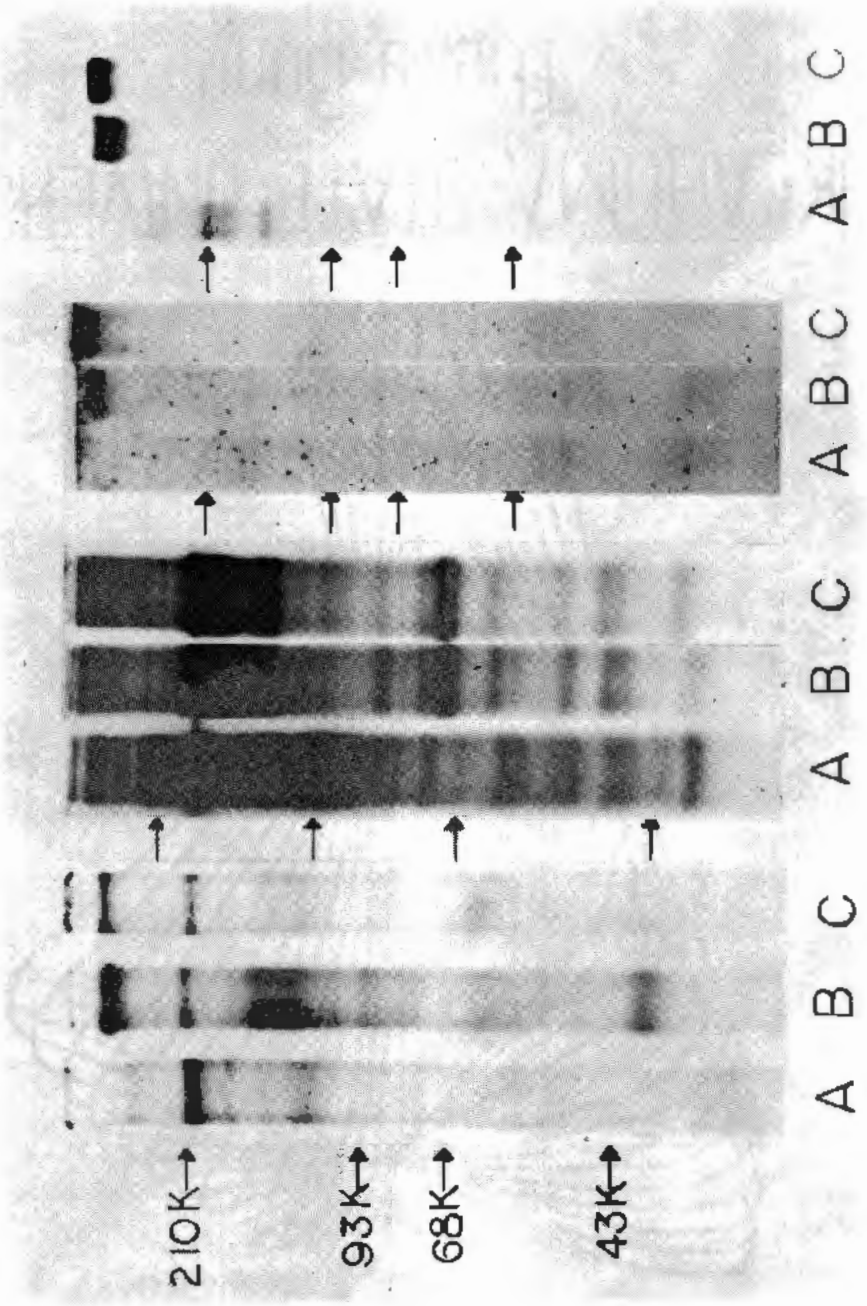

A B
A B
A B
A B
210K
93K
68K
43K

membranes are quite different.

Effect of Transplantation and Passage in Ascites Form on the Glycoproteins of 13762 MR Cells

13762 MR cells were transplanted into the peritoneal cavities of rats to give ascites tumors (MAT-MR1) which were passaged in vivo in ascites form. After the 16th passage the cells were analyzed by cell surface and metabolic labeling and for PNA and Con A receptors. For comparison MR and MAT-B1 cells were labeled under the same conditions. MAT-B1 was used because it is more stable under our passage conditions than MAT-B, from which both MAT-B1 and MR cells were derived. Moreover, the cell surface labeling patterns examined in these studies are not significantly different for MAT-B and MAT-B1 sublines. The results in Figure 5 show that substantial changes have occurred in the glycoproteins of the 13762 MR cells as a result of transplantation and passage in the ascites form. In particular, ASGP-1 is detectable by both metabolic labeling and PNA receptor analysis in the ascites cells (MAT-MR1-S) derived from transplanted MR cells. Thus the transplantation and passage have caused the MAT-MR1-S population to become more similar to the MAT-B1 cells than to the MR cells. The change was not limited to ASGP-1. The Con A receptors and ^{125}I-lactoperoxidase labeling patterns are both more similar for MAT-MR1-S and MAT-B1 cells than for MAT-MR1-S and MR cells. In addition the polypeptides of both cultured and ascites cells were examined by Coomassie blue staining of polyacrylamide gels run in SDS (Figure 3). The MAT-MR1-S ascites cells show more similarities to MAT-B1 ascites cells than they do to the MR cells (attached or spinner) from

Figure 5. Comparison of Labeling and Lectin Profiles of MR Spinner Culture Cells (A) to MR Transplanted into F344 Female Rats and Grown in Ascites Form, MAT-MR1-S (B), and to MAT-B1 Ascites Cells (C). Cells were labeled by lactoperoxidase-catalyzed ^{125}I-iodination (Panel I), [^{125}I]Con A staining (Panel II), [^{125}I]PNA staining (Panel III), and [^{3}H]glucosamine metabolic labeling (Panel IV). Panels III and IV are 4.5-15% acrylamide linear gradient slabs.


A B C A B C A B C A B C
210K
93K
68K
43K

which they were derived, particularly in the 43 K to 60 K region of the gel.

As shown previously (Huggins et al., 1980a), slow-migrating glycoproteins, which do not show a band for ASGP-1 by density gradient centrifugation, can be observed by glucosamine labeling and electrophoresis of the 13762 solid tumor. Therefore we examined the labeled component from MAT-MR1-S cells by density gradient centrifugation in CsCl and 4 M guanidine hydrochloride. A sharp peak was obtained at a density of 1.4 g/ml (Figure 6C), indicating that a glycoprotein with the density of ASGP-1 was responsible for the labeling of the MAT-MR1-S cells. By contrast the MR cells grown in culture (Figure 6D) do not show a defined glycoprotein peak by density gradient centrifugation.

Since neither the MR nor the 13762 solid tumor has ASGP-1, it was of interest to compare them. Substantial differences were found for both glucosamine labeling patterns and Con A receptors (Figure 7), although some of the Con A receptors, including the major one, appear to be the same. Thus the cell line adapted to cell culture from the ascites form does not represent simply a reversion to the form of the solid tumor, at least as far as glycoproteins are concerned.

Loss of ASGP-1 from Ascites Cells after Transfer to Cell Culture

To determine whether the changes in expression of ASGP-1 are reversible, MAT-MR1-S cells maintained by continuous passage in the ascites form were transferred to spinner culture. Analysis of PNA receptors ahowed that the level of ASGP-1 decreased with time in culture (Figure 8). Similar results were observed for MAT-B1 cells using either the PNA

Figure 6. Guanidine HCl and CsCl Density Gradient Profile of [^{3}H]Glucosamine Metabolically Labeled MAT-Bl Ascites Cells (A), 13762 Solid Tumor Glycoproteins (B), MAT-MRl-S Ascites Cells (C) and MR Cells Cultured Attached to the Substratum (D). Centrifugation was performed as previously described (Sherblom, Buck and Carraway, 1980a). Abscissa: elution weight (g); Ordinate: (left) cpm, (right) density (g/ml).

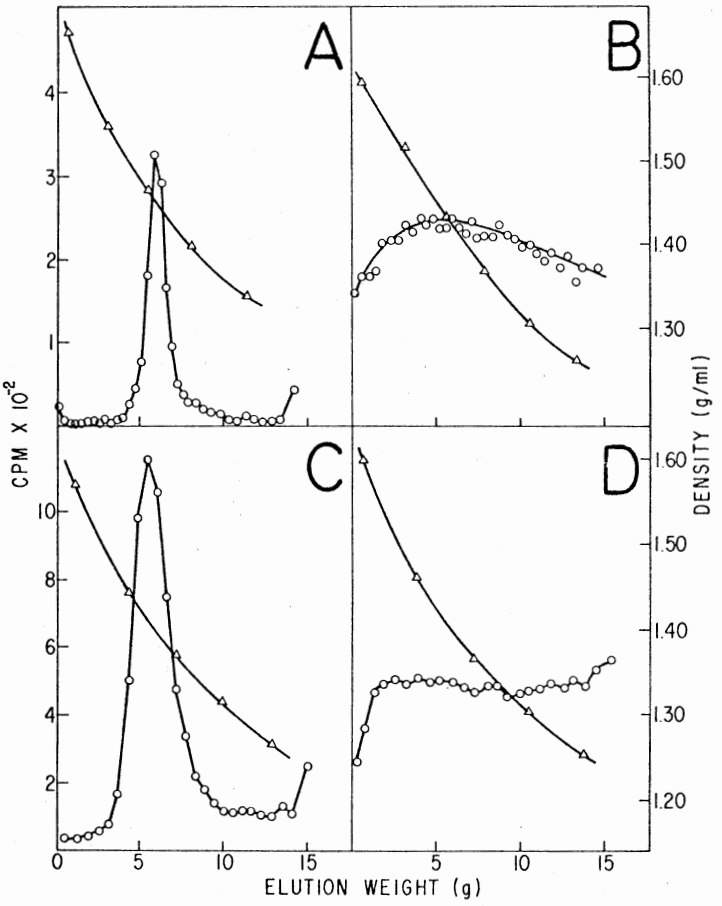
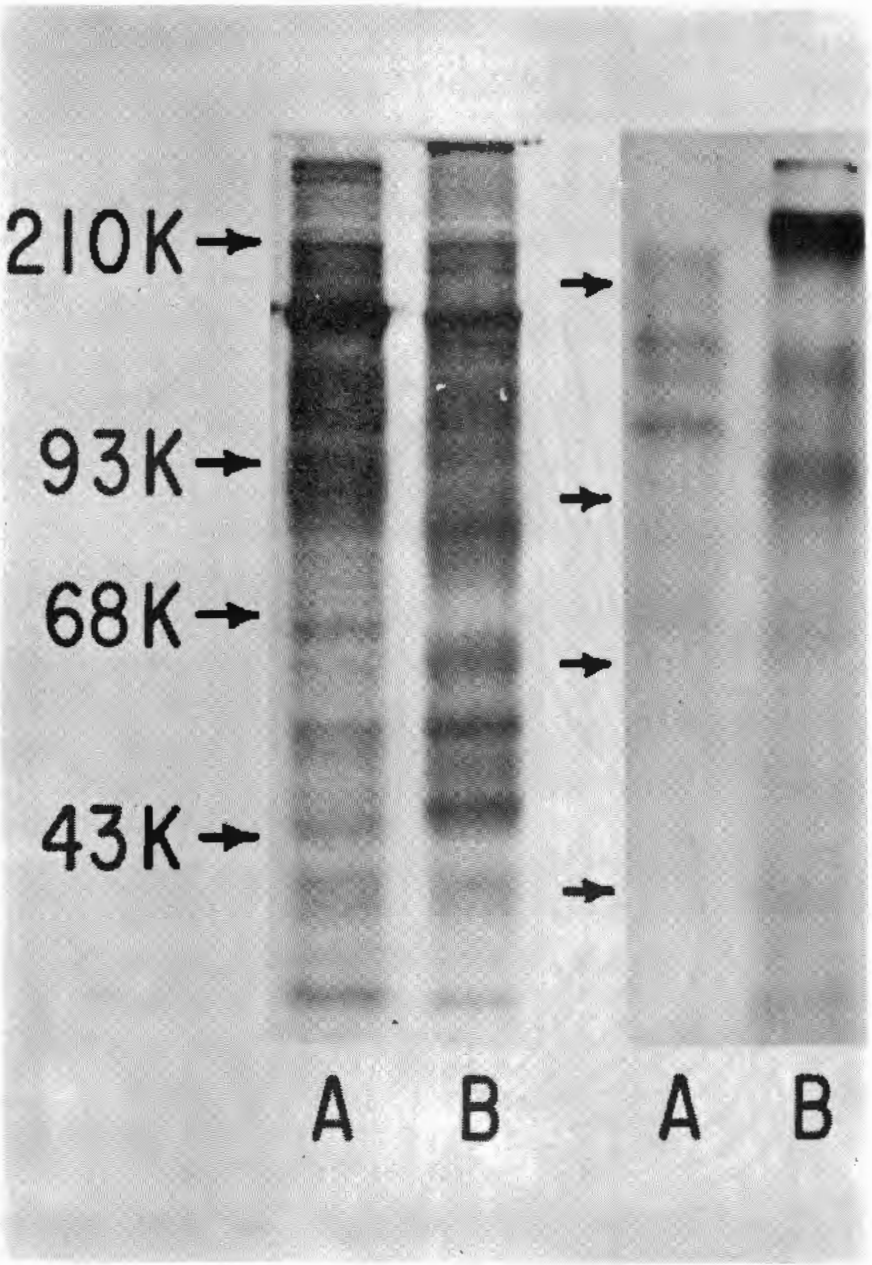

A
B
C
D
CPM X 10⁻²
DENSITY (g/ml)
ELUTION WEIGHT (g)
1.60
1.50
1.40
1.30
1.20
0
5
10
15

Figure 7. Comparison of Labeling Profiles of MR Spinner Culture Cells (A) to 13762 Solid Tumor (B) by [^{125}I]Concanavalin A Staining (Panel I) and [^{3}H]Glucosamine Metabolic Labeling (Panel II).


210K
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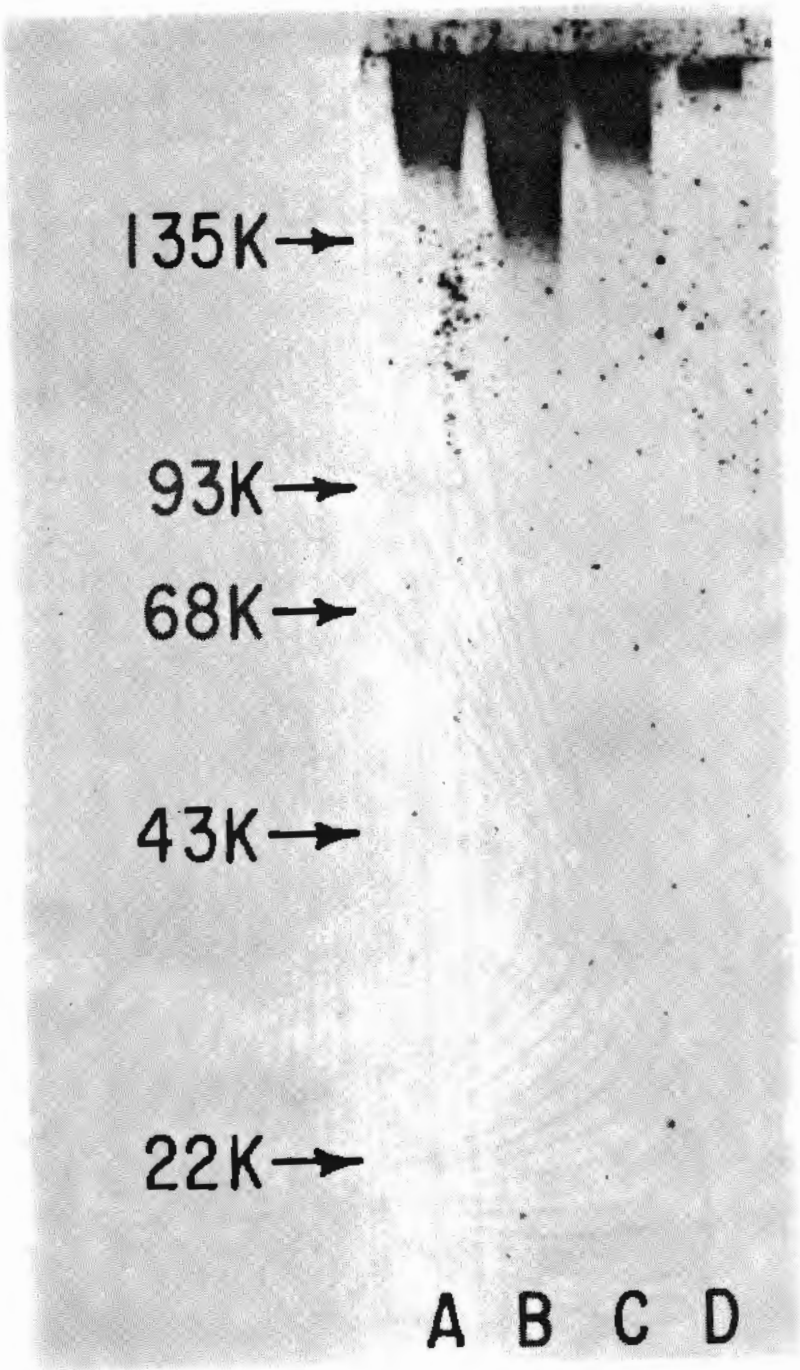
A
B
A
B

Figure 8. [^{125}I]PNA Staining of Slab Gels of SDS-Solubilized MAT-MR1-S Ascites Cells (A), and MR1-S Cells Cultured in Spinner (McCoy's 5A, Modified, plus 10% Fetal Calf Serum) 1.8 Days (B), 7.8 Days (C) and 64 Days (D). Gel is a 4.5-15% acrylamide linear gradient slab.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A B C D

receptor analysis (Figure 9) or glucosamine labeling (data not shown) to detect ASGP-1.

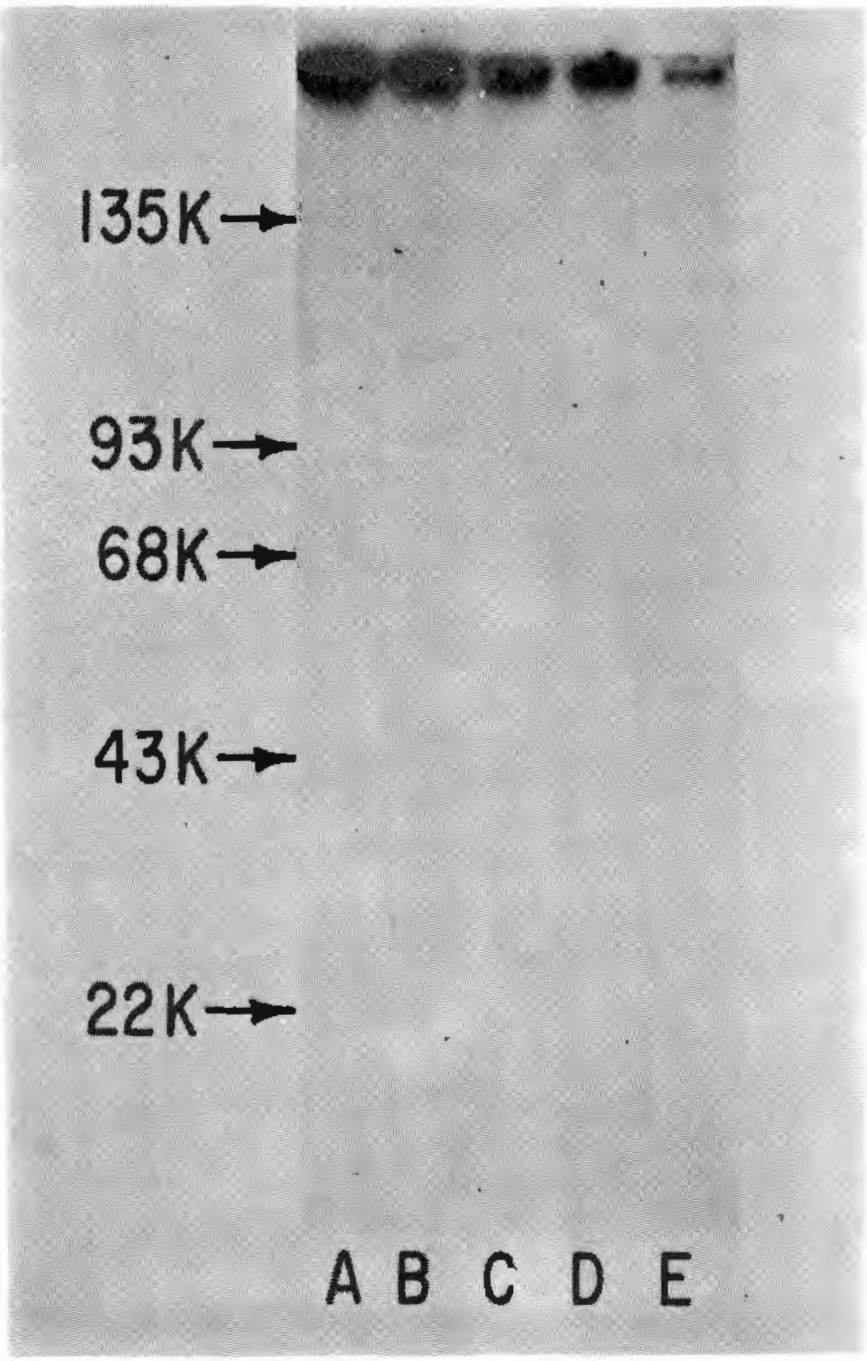
Expression of ASGP-1 during Early Passages in Ascites Form

The previous results indicate that expression of ASGP-1 as determined by glucosamine labeling or PNA receptor analysis is sensitive to the environment or growth state of the cells and suggest that ASGP-1 may be required for survival or growth in ascites form. For analysis of events occurring after transplantation, the MR cells were cloned. Several morphologically different clones were selected and transplanted. PNA receptor analyses of early in vivo passages of MR cells and the clones (in the ascites forms) showed an increase in a slow migrating band with an apparent molecular weight greater than 150,000 daltons (Figure 10). It was not identical to ASGP-1, however, because its electrophoretic mobility was different. Moreover glucosamine labeling did not show enhancement of this band in the ascites cells (Figure 11). ASGP-1 did not arise in the ascites forms derived from MR cells or its clones until later passages, including both morphological effects (Figure 13, note increased microvilli) and glycoprotein changes. MAT-B1 cells show somewhat similar morphological changes (in reverse) when transferred to cell culture (Figure 13). Moreover, a broad band at about 150 K observed by PNA receptor analysis (Figure 10) and glucosamine labeling (Figure 11) in the cultured cells is diminished or absent in the ascites cells.

Discussion

The present studies were undertaken to try to delineate some of the

Figure 9. [^{125}I]PNA Staining of Slab Gels of SDS-Solubilized B1 Cells in Spinner Culture (McCoy's 5A, Modified plus 25% Fetal Calf Serum) 5.5 Hours (A), 24 Hours (B), 47 Hours (C), 95 Hours (D) and 166 Hours (E). Slab is a 4.5-15% acrylamide linear gradient.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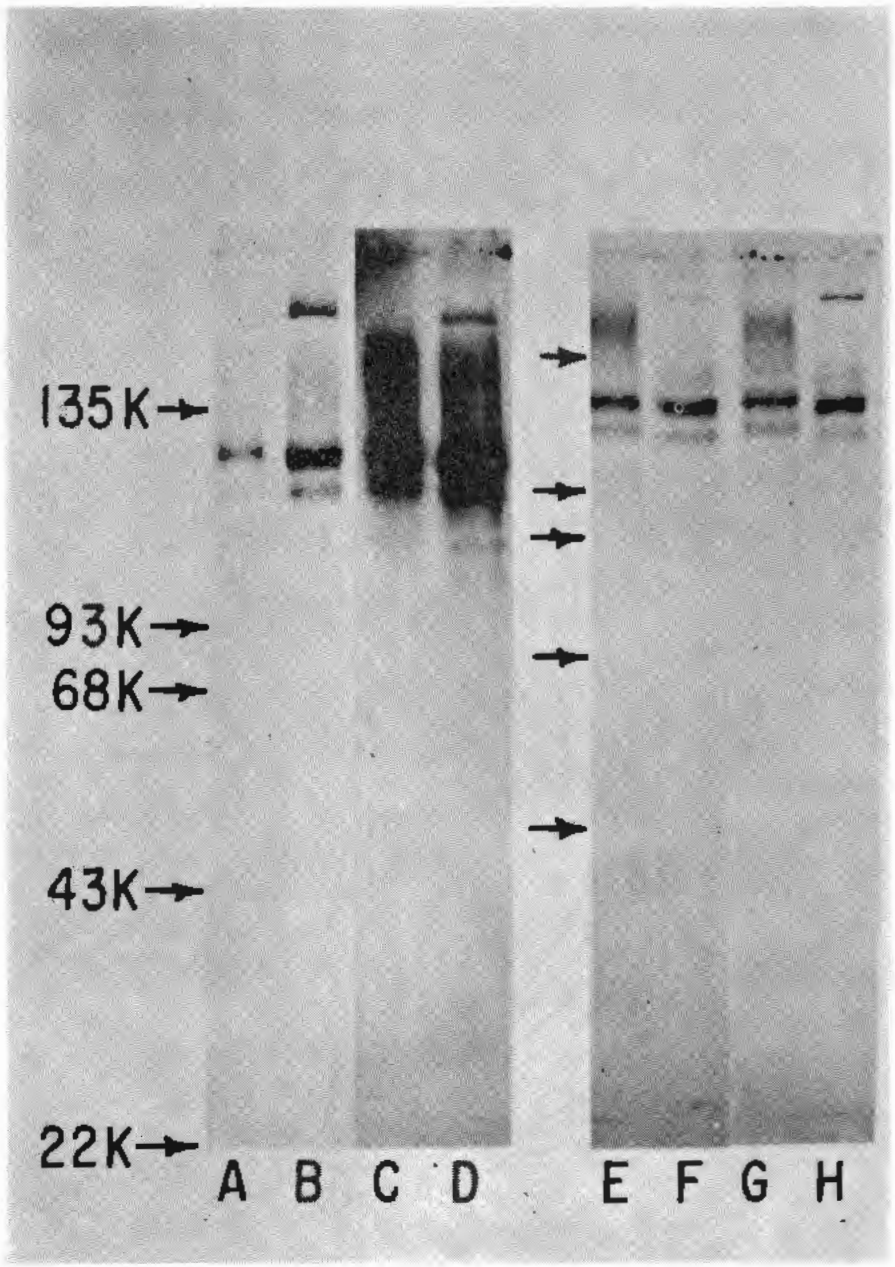
22K
A B C D E

Figure 10. [^{125}I]PNA Staining of Slab Gels of SDS-Solubilized MR Cells Maintained in Continuous Culture (A), the First Ascites Passage of the MR in F344 Rats (B), Clone 6 of MR in Culture (cMR6), Passage Number 4 (C), the First Ascites Passage of (C) in F344 Rats (D), Clone 9 of MR in Culture (cMR9), Passage Number 4 (E), the First Ascites Passage of (E) in F344 Rats (F), Clone 10 of MR in Culture (cMR10), Passage Number 4 (G), and the First Ascites Passage of (G) in F344 Rats (H). Electrophoreses were on 4.5-15% acrylamide gradient slabs.


135K
93K
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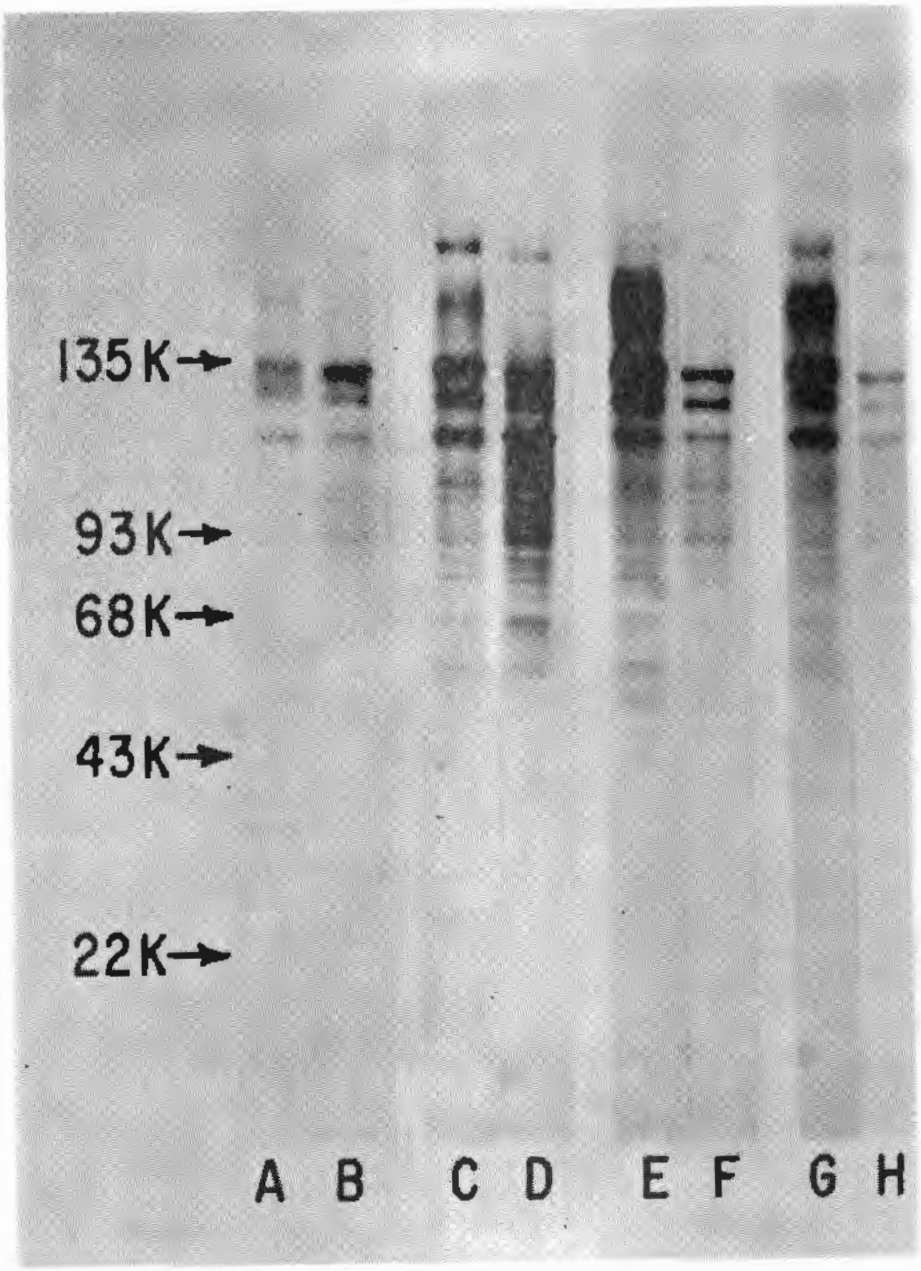
22K
A B C D E F G H

Figure 11. Comparison of SDS-Polyacrylamide Gel Profiles of [^{3}H]Glucosamine Metabolically Labeled Cells, MR Clone 6 (cMR6) Passage 4 *In Vitro* (A), the First Ascites Passage of (A) in F344 Rats (B), MR Clone 8 (cMR8) Passage 4 *In Vitro* (C), the First Ascites Passage of (C) in F344 Rats (D), MR Clone 9 (cMR9) Passage 4 *In Vitro* (E), the First Ascites Passage of (E) in F344 Rats (F), MR Clone 10 (cMR10) Passage 4 *In Vitro* (G), the First Asctites Passage of (G) in F344 Rats (H). Electrophoreses were on a 4.5-15% acrylamide linear gradient slab. Autoradiogram was exposed 34 days.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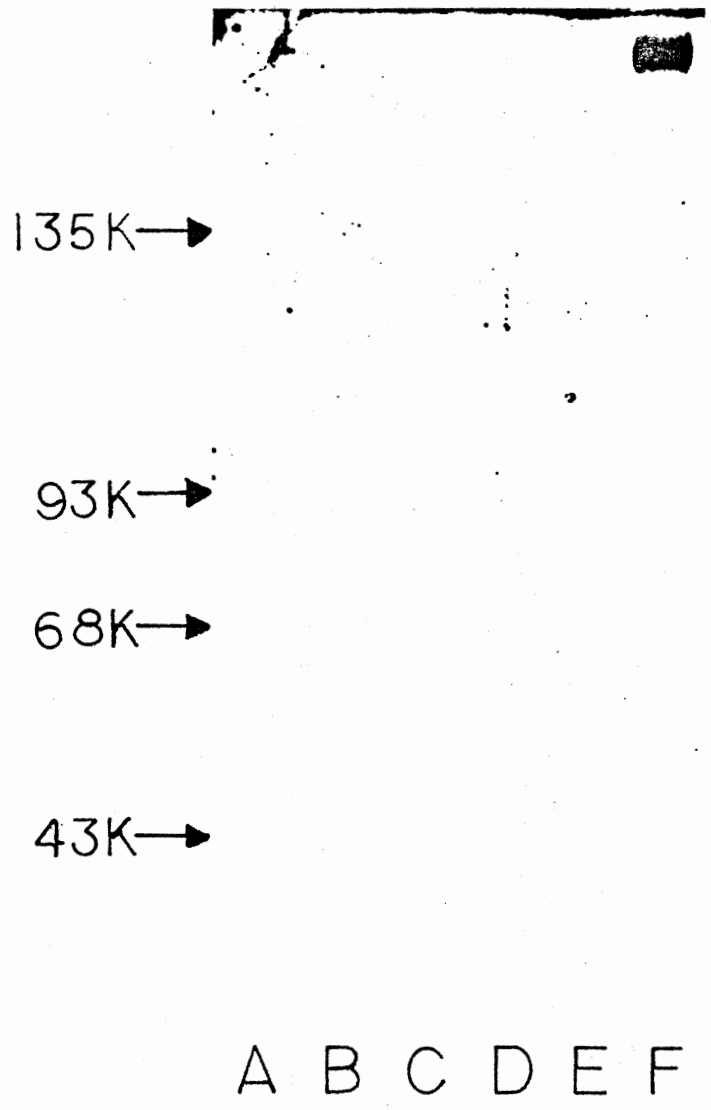
A B C D E F G H

Figure 12. [^{125}I]PNA Staining of Polyacrylamide Gels of SDS-Solubilized MR Clone 6 (cMR6) in Culture, Passage 4 (A), and Maintained as an Ascites Tumor (MAT-cMR6) in F344 Female Rats (B-F): Passage 1 (B), Passage 4 (C), Passage 6 (D), Passage 15 (E) and Passage 20 (F).


135K
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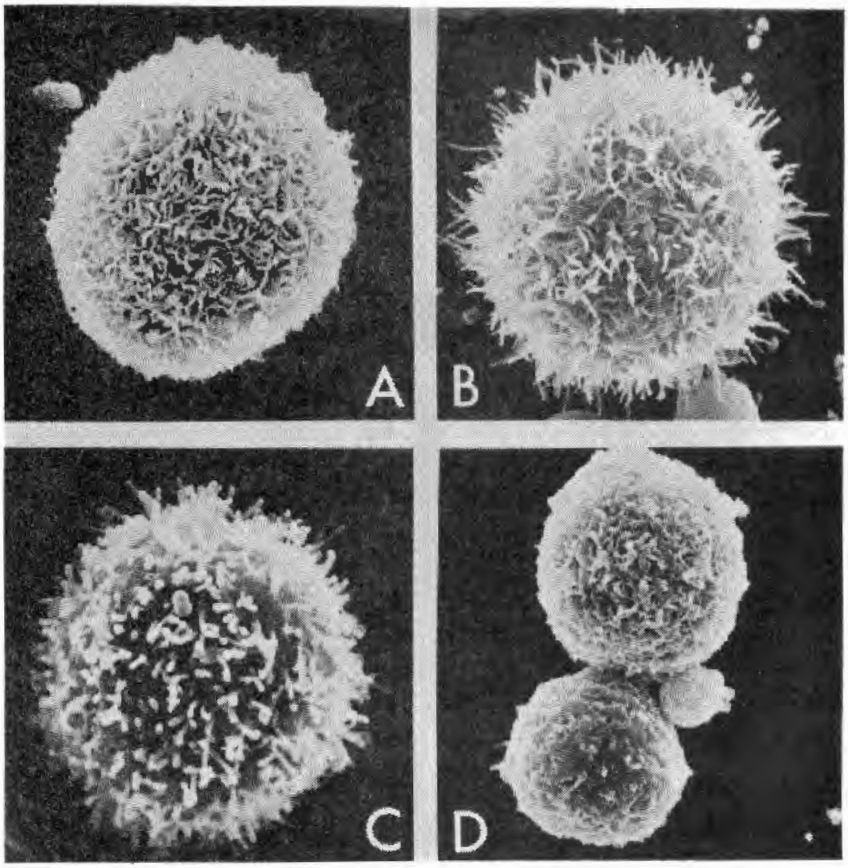
43K
A B C D E F

Figure 13. Scanning Electron Micrographs of MR Clone 6 (cMR6) In Vitro (A), and the Third Ascites Passage of cMR6 in F344 Rats (B), MAT-B1 Ascites Cells (C), and B1 Spinner Culture Cells after Culturing 7 Days (D). A, B and C are 3400 X and D is 3100 X magnification.


A
B
C
D

factors important in the expression of cell surface glycoproteins of 13762 tumor cells. We have concentrated on the major sialoglycoprotein ASGP-1 of the 13762 rat mammary adenocarcinoma ascites cells for several reasons: 1) It is the predominant glycoprotein of these cells, containing greater than 70% of the protein-bound label in cells metabolically labeled with glucosamine (Sherblom, Buck and Carraway, 1980a). 2) It is the only significant PNA receptor in the ascites cells, providing an additional specific assay (Sherblom, Buck and Carraway, 1980a). 3) It can also be identified by its position on CsCl density gradients in 4 M guanidine hydrochloride. 4) The glycoprotein has been recently purified (Sherblom, Buck and Carraway, 1980a) to homogeneity for further characterization and for antibody production. 5) ASGP-1 is not detected by the above techniques in either solid tumors (Huggins et al., 1980a) or in a cultured form of the tumor derived from an ascites subline.

This last result suggests that the presence of ASGP-1 might be required for ascites growth. That this is not the case was shown by the absence of ASGP-1 in early passages of the cultured tumor transplanted to give an ascites form. ASGP-1 did arise in later passages. Conversely, if the ascites tumor was transferred to culture or to a solid tumor by subcutaneous implantation, ASGP-1 disappeared over the period of several passages in the new form. Two likely explanations can be offered for this behavior: 1) The cells modulate their surface properties in response to some unknown stimulus or stimuli; or 2) the changes result from clonal selection of a population of the cells, and growth of cells without ASGP-1 is favored in the solid and cultured forms of the tumor whereas growth of cells containing ASGP-1 is favored in the ascites form. Two observations favor the former interpretation: 1) The increase in ASGP-1 in the

ascites form does not appear to be a continuous process, but occurs somewhat abruptly at a later passage. Exactly what factors are involved is unclear. 2) The clones undergo early modifications similar to that observed for the tumor which has not been cloned. However, the length of time required for the change might allow genetic changes that could produce new forms which would undergo a positive selection (Fidler, 1978). The selection pressure required for loss of ASGP-1 is unknown. An understanding of the mechanism of cellular change would be facilitated by knowledge of the molecular changes. Loss of ASGP-1, as detected by PNA assay, glucosamine labeling and density gradient centrifugation, requires either the failure to synthesize the polypeptide of ASGP-1 or a major alteration in the carbohydrate content of the glycoprotein. Using antibody prepared against the polypeptide chain portion of ASGP-1, it should be possible to distinguish between these possibilities.

Somewhat similar behavior has been observed for the TA3 mammary adenocarcinoma. The major sialoglycoprotein disappears on transfer to cell culture (Cooper et al., 1977). There is also a decrease in the sialoglycoprotein upon subcutaneous transplantation to give a solid tumor. However, in contrast to results with the 13762 tumor, the sialoglycoprotein increases with increased passage in the solid form until it approximates the level in the ascites form. Cooper et al. (1977) have suggested that the sialoglycoproteins of the TA3 tumor may contribute to the metastatic potential of these cells. The clones derived from the 13762 MR tumor should provide a system for testing the relationship between metastasis and the expression of ASGP-1.

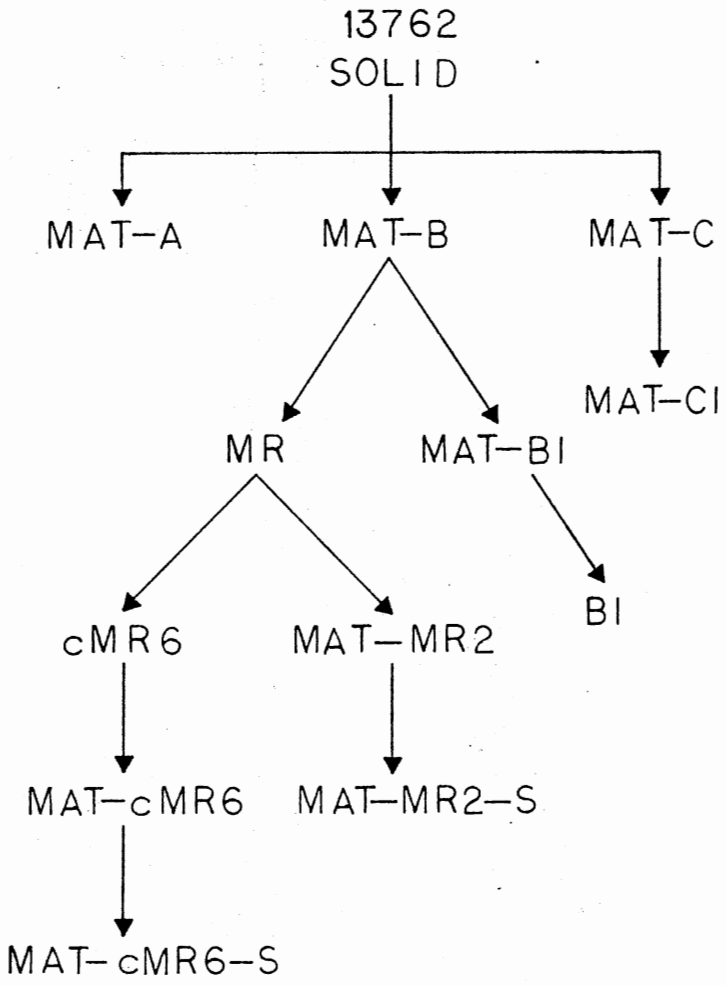
CHAPTER III

CHARACTERIZATION OF TWO NEW 13762 VARIANT ASCITES SUBLINES

Introduction

At least five 13762 rat mammary adenocarcinoma sublines (MAT-A, MAT-B, MAT-B1, MAT-C, MAT-C1) have been previously described (Carraway et al., 1978; Sherblom et al., 1980c; Sherblom, Buck and Carraway, 1980a). These five sublines (Figure 14) differ in surface morphology, Con A receptor redistribution, xenotransplantation and sugar composition of the oligosaccharide chains attached to the major cell surface glycoprotein, ASGP-1. The MAT-C and MAT-C1 cells have branched microvilli, no observable long-range lectin receptor redistribution and are transplantable across species barriers into mice. These sublines also contain large amounts of sialic acid and very little sulfate (Sherblom and Carraway, 1980b). In contrast, the MAT-B and MAT-B1 sublines have more normal-appearing non-branched microvilli, lectin receptor redistribution and are not transplantable across species barriers. These sublines contain approximately one-third as much sialic acid as the MAT-C1 line (Sherblom, Buck and Carraway, 1980a) and incorporate significant amounts of $[^{35}S]SO_4^{2-}$ into the glycoprotein ASGP-1. The MAT-A cells are similar to the MAT-B cells (Sherblom et al., 1980c; Carraway et al., 1978; Sherblom and Carraway, 1980b).

Figure 14. Schematic Diagram Showing the History of the 13762 Rat Mammary Adenocarcinoma Sublines.



MAT -ASCITES
MR -CULTURED
c -CLONED
S -SIALOGLYCOPROTEIN

Evidence is presented in Chapter II showing that when the 13762 cell culture line (13762 MR) is transplanted into F344 female rats, the sialoglycoprotein ASGP-1 develops after at least 16 passages. Two ascites tumor lines containing ASGP-1 were obtained in this manner and have been further characterized. The MAT-cMR6-S cells were derived from a recently cloned 13762 MR line whereas the MAT-MR2-S cells were derived from the MR 13762 cells maintained in continuous culture for approximately three years followed by one freeze-thaw. These new ascites lines differ from the previously described 13762 rat mammary adenocarcinoma variant ascites sublines.

Materials

D-1-[^{3}H]glucosamine HCl (2-6 Ci/mmole) and [^{35}S]sulfate (100 mCi/mmole) were from Amersham; Instagel was from Packard; CsCl (99.99%) was a product of Varlacoid; guanidine hydrochloride (Grade I) was from Sigma.

Ascites cells were maintained as previously described (Sherblom, Buck and Carraway, 1980a).

Methods

FITC-Con A lectin receptor redistribution, D-1-[^{3}H]glucosamine and [^{35}S]SO_4^{2-} incorporation *in vivo*, ASGP-1 purification and isopycnic density gradient centrifugation in CsCl with 4 M guanidine hydrochloride alkaline borohydride hydrolysis and Biogel P4 chromatography were performed as previously described (Huggins *et al.*, 1980b; Sherblom, Buck and Carraway, 1980a). Sialic acid release and assay were performed as described by Buck (1978). Scanning electron microscopy was performed as described by Carraway *et al.* (1979).

Xenotransplantation

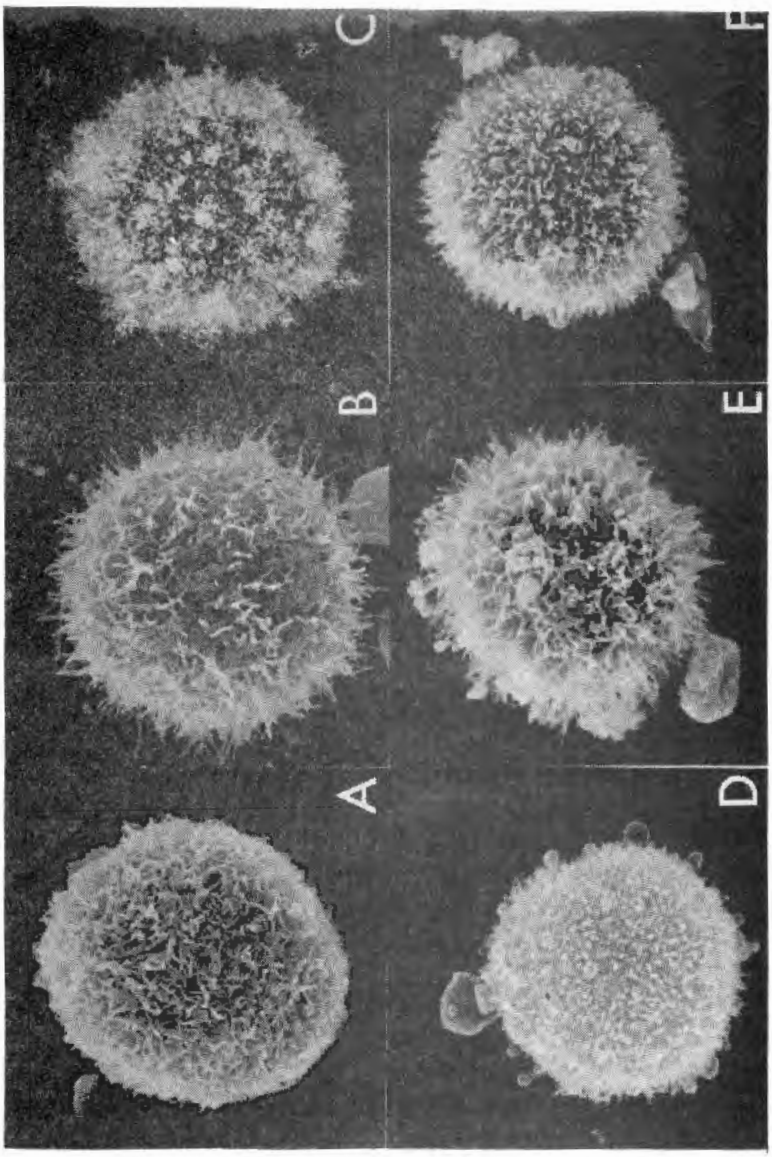
For each tumor line tested, eight C57Bl/6J female mice were injected with 1 x 10^6 tumor cells suspended in 0.25 ml of 0.9% saline, pH 7.4. Controls were injected with 0.25 ml saline.

Results

As shown in Figure 15, there are distinct morphological differences between early ascites passages (B,E) of the tumor cells compared to the cells maintained in culture (A,D). The cultured cells have an abundance of ruffles with no distinct microvilli. Blebbing occurs whether the cells are grown attached to a substratum or in spinner culture. The most prominent morphological alteration during early ascites passages (B,E) is the development of distinct cell surface microvilli. This represents an increase in the total membrane area exposed to the environment. However, in one case a much more pronounced morphological alteration is observed concommitant with or shortly after development of the large sialoglycoprotein ASGP-1 (C). Between the fifteenth and twenty-second passages, the MAT-cMR6-S microvilli developed a branched structure(C), similar to that observed in electron micrographs of the MAT-C1 cells (Carraway et al., 1978). Once again the total cell surface area has been increased. The MAT-MR2-S microvilli (F), although not branched, appear either as single structures or as clumps of microvilli attached to each other at the tips.

From previous studies on the MAT-C1 and MAT-B1 cells, the MAT-C1 Con A lectin receptor redistribution is greatly restricted whereas the MAT-B1 Con A receptors redistribute freely. Neither the MAT-cMR6-S

Figure 15. Scanning Electron Micrographs Showing the Progressive Morphological Changes as Cultured Cells Adapt to Ascites Growth. (A) cMR6 (MR clone 6) in in vitro culture, passage 4, (B) MAT-cMR6, ascites passage 3, (C) MAT-cMR6-S, ascites passage 22, after development of ASGP-1, (D) MR in in vitro culture attached to substratum, (E) MAT-MR2 ascites passage 13 and (F) MAT-MR2-S, ascites passage 28, after development of the glycoprotein. Magnification: A,B,D,E, 3400; C,F, 3100.

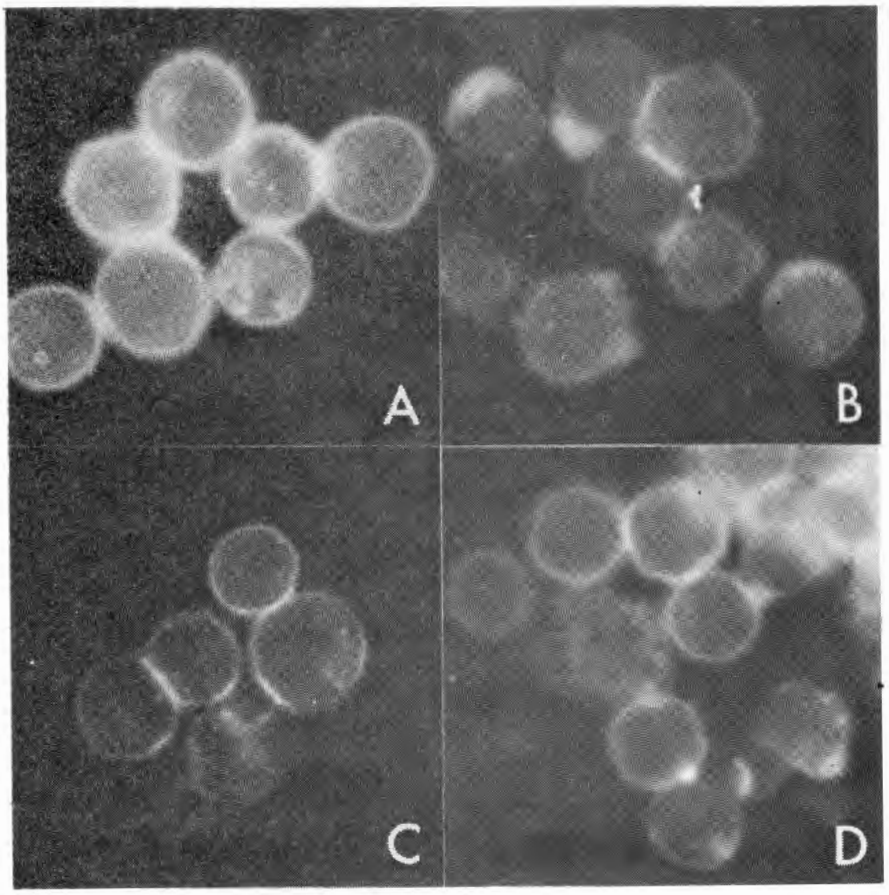

A
B
C
D
E
F

cells nor the MAT-MR2-S cells (Figure 16) show the large-scale Con A lectin receptor residtribution observed for MAT-B1 cells (Carraway et al., 1978). Nevertheless, minor lectin receptor mobility is apparent.

It has previously been suggested that the xenotransplantability difference observed between the MAT-B1 and MAT-C1 cells could be due to the differences observed in the oligosaccharide composition of ASGP-1 isolated from these sublines (Sherblom, Buck and Carraway, 1980a). Therefore, the ASGP-1 isolated from the MAT-cMR6-S and MAT-MR2-S ascites sublines was partially characterized in order to substantiate further or disprove this theory. Figure 17 shows that MAT-cMR6-S ASGP-1 migrates with a density of 1.401 in CsCl and 4 M guanidine hydrochloride and can be labeled using [^{3}H]glucosamine and [^{35}S]sulfate. Both of these properties have also been observed for the MAT-B1 ASGP-1. On the other hand, MAT-MR2-S ASGP-1 (Figure 18) has an apparent density of 1.410 and incorporates [^{3}H]glucosamine but scant, if any [^{35}S]sulfate. These properties are similar to MAT-C1 ASGP-1 (Sherblom, Buck and Carraway, 1980a).

As statedpreviously, the sugar composition of the MAT-B1 ASGP-1 oligosaccharide side chains differs from MAT-C1. Following ASGP-1 purification, the O-linked oligosaccharide side chains are released from the protein core using mild alkaline-borohydride hydrolysis. Chromatography over Biogel P4 separates the released oligosaccharide side chains according to size. Two major oligosaccharide peaks are observed for MAT-C1 ASGP-1 labeled in vivo with [^{3}H]glucosamine and three for MAT-B1 ASGP-1 (Sherblom, Buck and Carraway, 1980a). The Biogel P4 profile (Figure 19A) of the oligosaccharides released from [^{3}H]glucosamine labeled MAT-cMR6-S ASGP-1 provides further evidence that the MAT-cMR6-S

Figure 16. Dark-Field Micrographs of FITC-Con A Labeled Prefixed MAT-cMR6-S Cells Passage 25 (A), MAT-cMR6-S Cells Treated with 1 mg/ml FITC-Con A in DPBS with 1 mg/ml Glucose, pH 7.4 30 min at 37^{o} Followed by Fixation in 1% Glutaraldehyde (B), MAT-MR2-S Passage 22 Prefixed Cells (C) and MAT-MR2-S Cells Treated with FITC-Con A before Fixation.


A
B
C
D

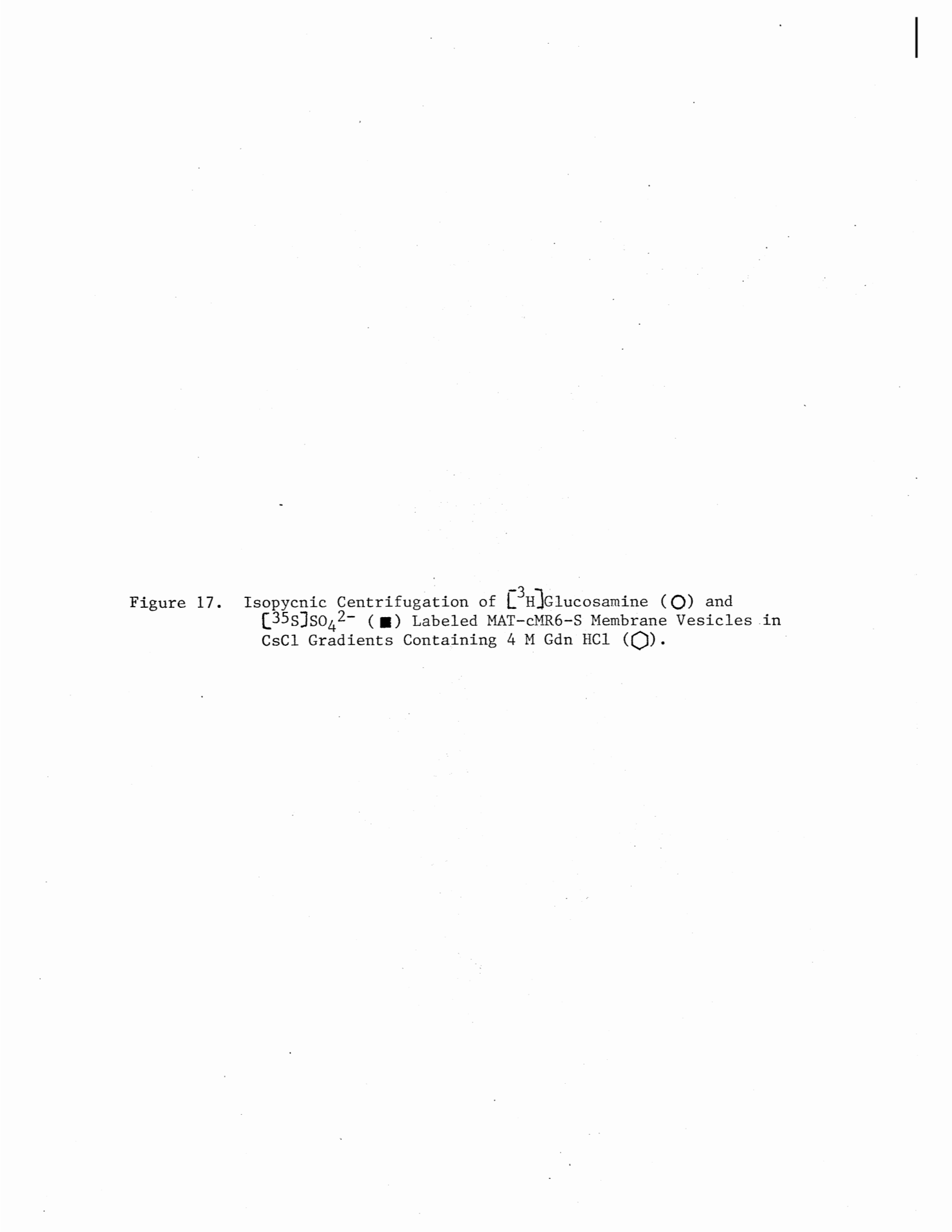


Figure 17. Isopycnic Centrifugation of [^{3}H]Glucosamine (○) and [^{35}S]SO_4^{2-} (■) Labeled MAT-cMR6-S Membrane Vesicles in CsCl Gradients Containing 4 M Gdn HCl (⬡).

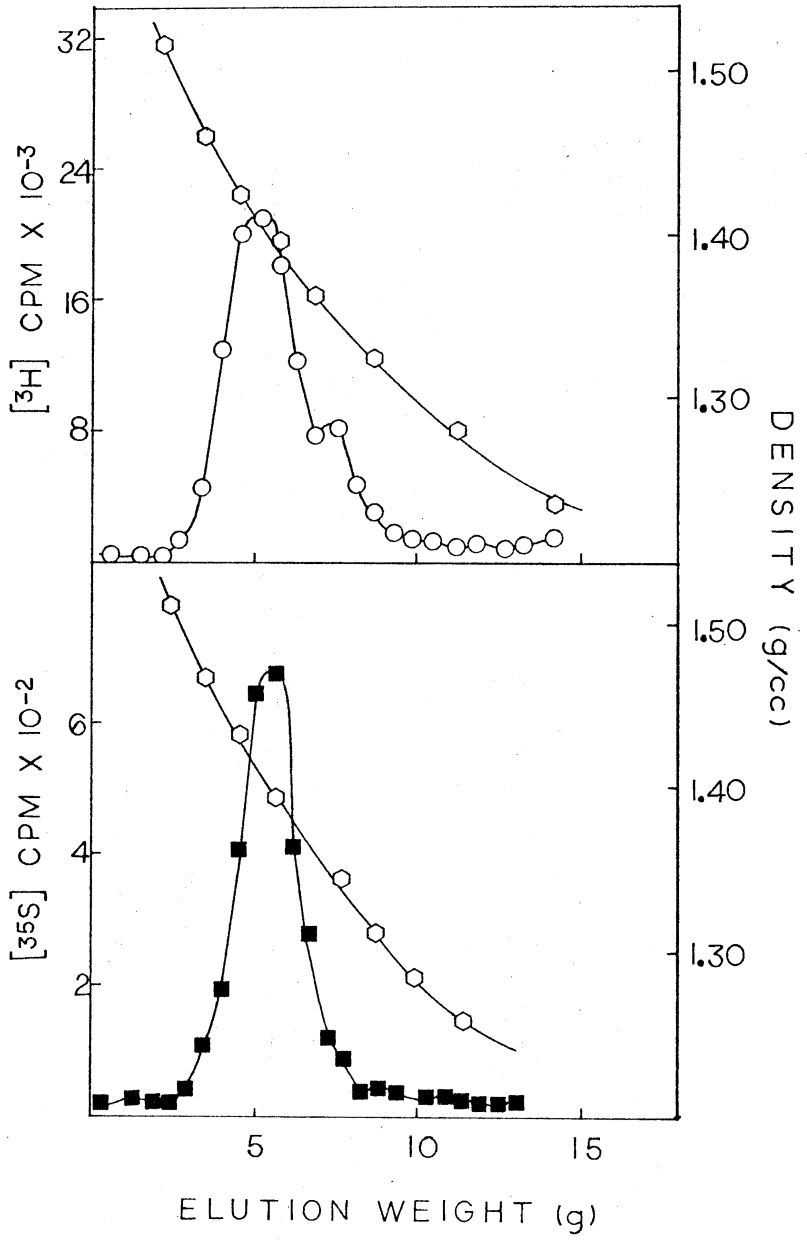

32
24
16
8
[3H] CPM X 10-3
1.50
1.40
1.30
DENSITY (g/cc)
6
4
2
[35S] CPM X 10-2
1.50
1.40
1.30
5
10
15
ELUTION WEIGHT (g)

Figure 18. Isopycnic Centrifugation of [^{3}H]Glucosamine (○) and [^{35}S]SO_4^{2-} (■) Labeled MAT-MR2-S Membrane Vesicles in CsCl Gradients Containing 4 M Gdn HCl (⬡).

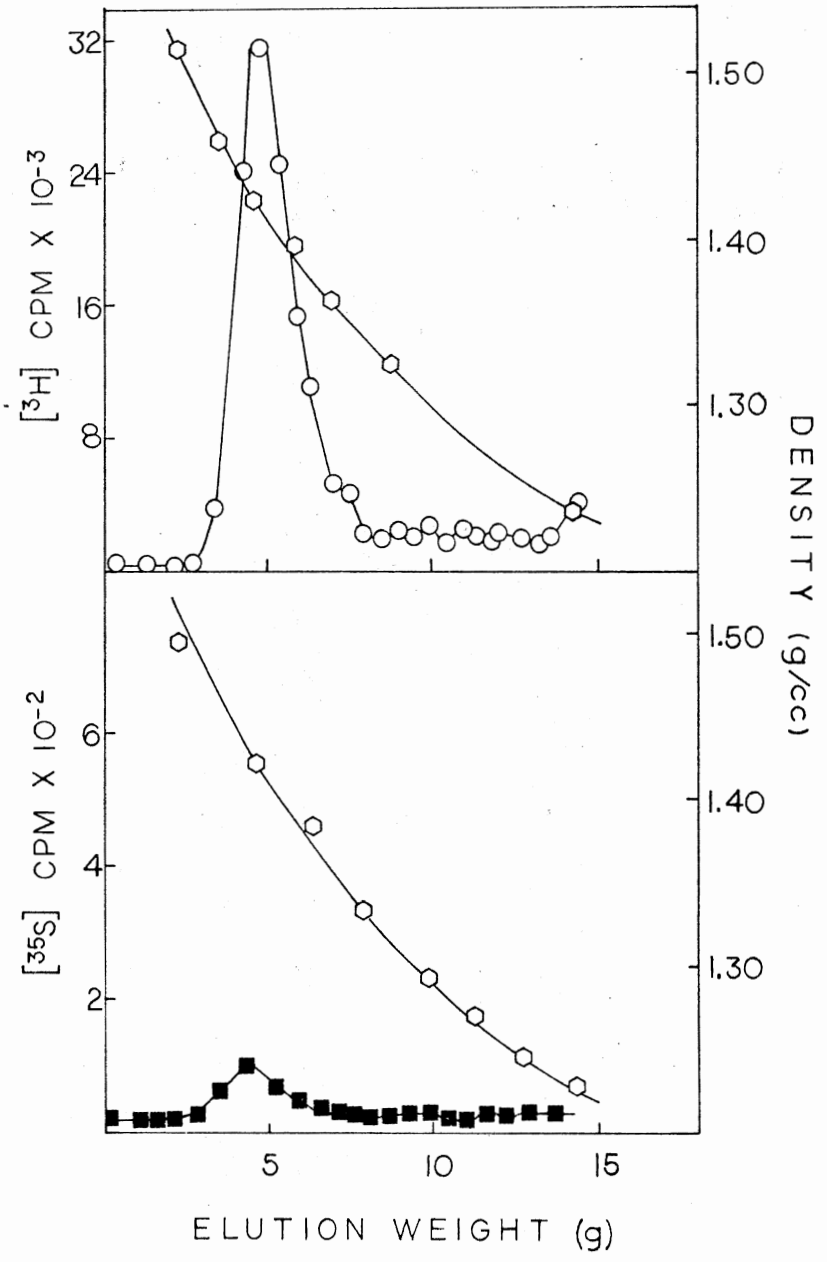
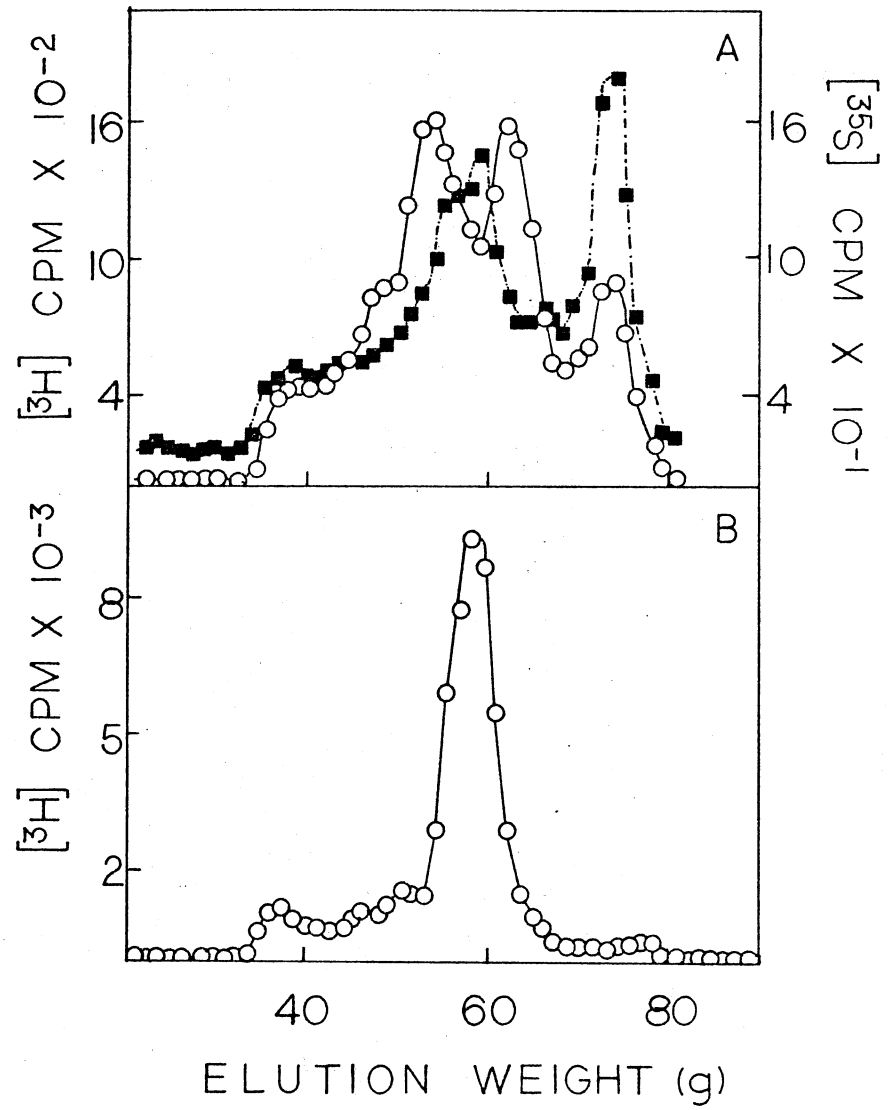

32
24
16
8
[3H] CPM X 10-3
1.50
1.40
1.30
DENSITY (g/cc)
6
4
2
[35S] CPM X 10-2
1.50
1.40
1.30
5
10
15
ELUTION WEIGHT (g)

Figure 19. Gel Filtration of MAT-cMR6-S (A) and MAT-MR2-S (B) ASGP-1 Products on Biogel P4 Following Alkaline-Borohydride Treatment (1.0 M $NaBH_4$, 0.05 M NaOH, 16 hours, 45°). The samples were mixed, alkaline-borohydride treated, neutralized with acetic acid and chromatographed on a Biogel P4 column (200-400 mesh, 0.9 x 113 cm) equilibrated with 1.0 M pyridine-acetate, pH 6.0. Fractions were assayed for determination of ^{3}H (○) and ^{35}S (■) radioactivity.


A
B
[3H] CPM X 10-2
[3H] CPM X 10-3
[35S] CPM X 10-1
16
10
4
8
5
2
40
60
80
ELUTION WEIGHT (g)

ASGP-1 is very similar to MAT-B1 ASGP-1 (Sherblom, Buck and Carraway, 1980a). However, the oligosaccharide composition of the MAT-MR2-S ASGP-1 (Figure 19B) is even simpler than that observed for MAT-C1 ASGP-1. Only one major oligosaccharide peak is observed.

Although the specific sugar composition of the oligosaccharide side chains has not been determined, acid hydrolysis releases only slightly less sialic acid from MAT-MR2-S cells than from MAT-C1 cells. Once again, comparable amounts of sialic acid are released from both MAT-cMR6-S and MAT-B1 cells (Table I).

During a xenotransplantation study of the MAT-cMR6-S and MAT-MR2-S cells into C57BL/6J mice, 6 of 8 mice injected with 1×10^6 MAT-cMR6-S cells died due to ascites tumors within 30 days. At the end of 90 days none of the mice injected with MAT-MR2-S ascites cells, the 13762 MR cell culture cells or the saline controls had died.

Thus, the MAT-cMR6-S cells exhibit a surface morphology similar to the MAT-C1 cells, have a degree of Con A lectin receptor redistribution intermediate between MAT-C1 and MAT-B1 cells and are xenotransplantable. However, MAT-cMR6-S ASGP-1 resembles MAT-B1 ASGP-1 in density, chromatographic profile of the oligosaccharides, $[^{35}S]$sulfate incorporation and acid hydrolyzable sialic acid.

In contrast, MAT-MR2-S cells have a surface morphology more like the MAT-B1 cells, although some of the MAT-MR2-S microvilli appear to be agglutinated or cross-linked at the tips. The MAT-MR2-S cells show much less Con A lectin receptor redistribution than MAT-B1 cells and are not xenotransplantable under the conditions used. However, MAT-MR2-S ASGP-1 exhibits a density and sialic acid composition similar to MAT-C1 ASGP-1. MAT-MR2-S ASGP-1 does differ appreciably from MAT-C1 ASGP-1 with respect

TABLE I

SUMMARY OF PROPERTIES OF MAT-MR2-S, MAT-cMR6-S, MAT-B1 AND MAT-C1 ASCITES 13762 RAT MAMMARY ADENOCARCINOMAS

Property	MAT-MR2-S	MAT-cMR6-S	MAT-B1	MAT-C1
[^{125}I]PNA Binding	(+)	(+++)	(+++)	(++)
Sulfation	(+,-)	(+)	(+)	(-)
Morphology of Microvilli	Not Branched	Branched	Not Branched	Branched
[^{125}I]Con A Receptor Redistribution	(-)	(+,-)	(+)	(-)
nmoles Sialic Acid Per 1 x 10^7 Cells	28	13	16	37
ASGP-1 Density	1.41	1.40	1.40	1.41
Number of Major ASGP-1 Oligosaccharides	1	3	3	2
Xenotransplantation	(-)	(+)	(-)	(+)

to the oligosaccharide profile observed on Biogel P4.

Discussion

The results summarized above indicate that the xenotransplantation difference Carraway et al. have observed between the MAT-B1 and MAT-C1 ascites cells (Carraway et al., 1978; Sherblom, Buck and Carraway, 1980a) cannot be attributed solely to the variation observed in the ASGP-1 oligosaccharides since the MAT-cMR6-S cells have a surface morphology similar to the MAT-C1 cells by scanning electron microscopy, an ASGP-1 very similar to MAT-B1 ASGP-1 and are xenotransplantable.

However, it is possible that the microvillar structure is a critical factor in determining xenotransplantability. Since ASGP-1 is a major component of the microvilli (Howard et al., submitted for publication), ASGP-1 may be one determinant of the microvillar structure. Nevertheless, the microvilli are comprised of a number of proteins as well as membrane lipids. Alteration or absence of any critical microvillar structural component could alter the microvillar morphology observed. Thus, the MAT-B1 cells could lack a different microvillar structural component critical for formation of branched microvilli.

CHAPTER IV

RELEASE OF CELL SURFACE MATERIAL FROM MAT-B1 AND MAT-C1 ASCITES RAT MAMMARY ADENOCARCINOMA CELLS

Introduction

Cell surface glycoproteins are believed to play important roles in allowing the escape of tumor cells from destruction by the immune system of the host (Nicolson and Poste, 1976). It has been postulated that the shedding of cell surface antigens and other glycoproteins from the tumor may block destruction of the tumor cells by cells of the immune system (Nicolson and Poste, 1976; Hellstrom and Hellstrom, 1974; Cooper, Codington and Brown, 1974; Kim et al., 1975), but as yet there is relatively little information concerning the mechanisms by which cell surface material is shed. Miller et al.(1978a,b) have investigated the kinetics of release of glucosamine-labeled, perchloric acid-soluble components from the cell surface of the allo- and xenotransplantable mouse ascites Ta3-Ha mammary adenocarcinoma. Vicia graminea receptors derived from the major cell surface sialoglycoprotein(s) epiglycanin were released in a biphasic manner. However, several questions remain unanswered concerning the mechanisms of release of cell surface components. Since the native form(s) of epiglycanin was (were) not characterized, it is not possible to compare the released and cell-associated forms of the

glycoprotein. The fact that the released material is soluble suggests an alteration in the glycoprotein is occurring with the release. Moreover, such analyses ignore possible contributions of shed, insoluble fractions. Shedding of membrane fragments has been observed with mammary carcinomas (Nordquist, Anglin and Lerner, 1977) and other cells (Van Blitterswijk et al., 1977) and may be an intermediate in the release of soluble surface material.

For our investigations of these questions we have used the MAT-B1 and MAT-C1 ascites sublines of the 13762 rat mammary adenocarcinoma. These are similar to the St and Ha sublines of the TA3 adenocarcinoma in many respects, but offer several advantages. Although the MAT-C1 subline is xenotransplantable and MAT-B1 is not, both have large amounts of a single major cell surface sialoglycoprotein termed ASGP-1 (Buck, Sherblom and Carraway, 1979; Sherblom et al., 1980c; Sherblom, Buck and Carraway, 1980a). This contrasts with the TA3 sublines in which the allo- and xenotransplantable Ha subline has epiglycanin, but the nontransplantable St subline does not (Codington et al., 1975; Miller, Hay and Codington, 1977). Moreover, the ASGP-1 from both MAT-B1 and MAT-C1 sublines has been purified to homogeneity and shown to be the same size as the glycoprotein solubilized directly in SDS from the corresponding cells (Sherblom, Buck and Carraway, 1980a). Amino acid compositions from MAT-B1 and MAT-C1 were very similar, but their carbohydrate compositions, molecular weights and oligosaccharides were different (Sherblom, Buck and Carraway, 1980a). During in vivo or in vitro metabolic labeling each subline incorporates greater than 70% of protein-bound glucosamine label into ASGP-1 (Sherblom et al., 1980c; Sherblom, Buck and Carraway, 1980a). Finally, previous studies have

demonstrated the release of particulate cell surface fractions, including microvilli (Huggins et al., 1980b), from these cells, and procedures have been developed for fractionating these materials (Carraway et al., 1980; Huggins et al., 1980b).

The current studies set out to answer the following questions. What are the differences between the shed soluble and native membrane-bound forms of ASGP-1 that might relate to the mechanism of shedding? What is the nature of the particulate material shed in vitro and in vivo? What are the rates of shedding of soluble and particulate fractions for the two sublines? How do the amounts and types of materials shed from the two sublines compare? Using the metabolic labeling and fractionation of released cellular components on Percoll gradients or by differential centrifugation we have found that ASGP-1 is released on microvilli or membrane fragments and in soluble form. The soluble form, after denaturation in dodecyl sulfate, appears smaller by gel filtration than purified ASGP-1 or ASGP-1 from shed microvilli (Howard et al., submitted for publication), suggesting that proteolysis may be involved in its release. Release of the soluble ASGP-1 is decreased by the protease inhibitor Aprotinin or EDTA, providing further evidence for a proteolytic cleavage mechanism of release. Examination of ascites fluid showed similar types of shed material to those found in vitro. The rates of release of cell surface material in vitro were similar for MAT-C1 and MAT-B1 cells. The amount of soluble ASGP-1 in ascites fluid was 2.5 times greater for MAT-C1 than MAT-B1 cells, but the amount of ASGP-1 in the ascites fluid in insoluble form was slightly less for MAT-C1 than MAT-B1 cells. Although the increased amount of soluble ascites fluid ASGP-1 for MAT-C1 cells correlates with the xenotransplantability, it is doubtful whether this

quantitative difference in shedding can be responsible for the transplantation differences.

Materials

McCoy's 5A (modified), nonessential amino acids, fetal calf serum, EDTA and penicillin/streptomycin solution were from Gibco, sodium pyruvate was from Microbiological Associates, PPO (2,5-diphenyloxazole), soybean trypsin inhibitor, trypsin, actinomycin D, cycloheximide, puromycin, colchicine, cytochalasin D, theophylline, sodium azide, 2-deoxyglucose, PMSF (phenylmethyl sulfonyl fluoride), Aprotinin and EGTA were from Sigma, D-1-[^{3}H]glucosamine HCl (2-6 Ci/mmole), [^{3}H]leucine (55 Ci/mmole) and carrier-free [^{125}I]NaI were from Amersham/Searle. Instagel was from Packard, Percoll was from Pharmacia, polycarbonate filters were from Bio-Rad, peanut lectin was from Miles-Yeda, Ltd. and calcium ionophore A23187 was a gift from Eli Lilly.

The 13762 ascites tumors (Carraway et al., 1978) were passaged as described previously (Sherblom et al., 1980c).

Methods

Characterization of Shedding In Vitro

MAT-B1 or MAT-C1 tumor-bearing rats were injected with 0.1 mCi [^{3}H]glucosamine 16-17 hours before sacrifice (Sherblom et al., 1980c; Sherblom, Buck and Carraway, 1980a). Tumor cells were removed aseptically and washed three times with McCoy's 5A (modified) culture medium to which 25% fetal calf serum and 10 μg/ml Gentamicin had been added. Cells were resuspended in fresh medium with fetal calf serum and incubated in spinner culture at 37^o in a 5% CO_2 atmosphere for various times. For pulse-chase

analysis of the kinetics of shedding the incubations contained 3.8×10^{-3} μmole/ml cold gulcosamine. Insoluble fractions were separated from cells using a 30% Percoll self-forming gradient. Soluble ASGP-1 was obtained after pelleting cells at 121 g for 15 min by filtering through 0.1 μ pore size polycarbonate membranes followed by dialysis against water or by centrifuging at 100,000 g for 1.5 hours.

Radioactive fractions were counted in 10 ml Instagel in a Tri-Carb liquid scintillation counter. Electrophoresis in SDS on 4.5-15% gradient polyacrylamide gels was performed as previously described (Sherblom, Buck and Carraway, 1980a).

Trypsin Treatment of MAT-B1 Cells

Labeled, washed MAT-B1 cells, obtained as described above, were resuspended in complete medium with 1 mg/ml glucose, then treated with 50 μg/ml trypsin for 30 min in DPBS. Soybean trypsin inhibitor was added (100 μg/ml) for an additional 30 min, and the cells were washed and used for a shedding experiment.

Effect of Perturbants on In Vitro Shedding

Experiments were performed as described above, using a two hour incubation, except that the effectors were included at the concentrations noted. Effectors (and concentrations) used were: soybean trypsin inhibitor (1 mM), phenylmethyl sulfonyl fluoride (1 mM), colchicine (1 mM), EGTA (1 mM), ionophore A23187 (0.5 mM) with and without 1 mM $CaCl_2$, theophylline (1 mM), cytochalasin D (1 mM), sodium azide (1 mM), actinomycin D (0.1 mM), puromycin (0.1 mM), cycloheximide (1 mM), Aprotinin (20 Kiu/ml), EDTA (1 mM), deoxyglucose (1 mM) and dibucaine (1 mM).

Characterization of Material Shed In Vivo

The labeled cell suspension was aspirated from the rat peritoneal cavity without dilution and cells were pelleted at 480 g for 5 min. Particulate and soluble fractions were obtained by Percoll gradient and differential centrifugation, respectively, as described above. Protein was quantitated by the method of Lowry et al. (1951). Other characterization procedures were performed as described above.

Incorporation of [^{3}H]Leucine

Ascites cells were removed aseptically from the peritoneal cavity of MAT-B1 and MAT-C1 tumor-bearing F344 female rats. Cells were washed two times using McCoy's 5A (modified) media, pelleted at low speed in a clinical centrifuge, then resuspended at approximately 6-7 x 10^6 cells per ml in media supplemented with 25% fetal calf serum and 10 μg/ml Gentamicin antibiotic. Spinner cultures were grown in a 5% CO_2 atmosphere for the remainder of the experiment. [^{3}H]leucine (6.7 μCi/ml) was injected and sampling begun. At each time point, three 0.1 ml aliquots were heat denatured onto GF/A glass fiber filters, soaked in 10% TCA, washed using 5% TCA followed by ice cold water, solubilized in 0.5 ml Soluene 350 and counted for radioactivity using 10 ml Instagel solvent. At the same time, a 1.0 ml aliquot of the cell suspension was diluted to 5 mls with a Percoll-DPBS mixture giving a final Percoll concentration of 8% for MAT-B1 cells and 11% for MAT-C1 cells. Cells were pelleted at 2000 rpm for 5 min, then solubilized in electrophoresis sample buffer containing 3% SDS and mercaptoethanol at 100° 10 min. 1.5 x 10^5 cells per well were electrophoresed on a 4.5-15% acrylamide gradient slab. The

ASGP-1 and actin bands were sliced out using a razor blade, solubilized in 0.3 ml Soluene 350 and counted for radioactivity using 5 ml Instagel solvent.

Quantitation of Soluble ASGP-1 in Ascites Fluid

ASGP-1 was purified from MAT-B1 and MAT-C1 cells as previously described (Sherblom, Buck and Carraway, 1980a). Various amounts of the glycoproteins (0.02-1.5 μg) were applied to 4.5-15% gradient polyacrylamide gels and electrophoresed in SDS. The gels were reacted with ^{125}I-labeled peanut agglutinin, which reacts specifically with ASGP-1 (Huggins et al., 1980a). The labeled glycoprotein-lectin bands were cut from the gels and counted to prepare a standard curve, which was linear for each glycoprotein over the range of protein concentrations used. Samples of the soluble fraction of ascites fluid were subjected to the same procedure, permitting determination of the concentrations of ASGP-1 in each ascites fluid sample.

Results

Characterization of Shedding In Vitro

For characterization of the shed material, glycoproteins of MAT-B1 and MAT-C1 cells were labeled by injection of [^{3}H]glucosamine into the peritoneal cavity. As shown previously (Buck, Sherblom and Carraway, 1979; Sherblom, Buck and Carraway, 1980a; Sherblom et al., 1980c), the protein-bound label is found predominantly in one component, ASGP-1, illustrated in Figures 20 and 21 for MAT-B1 and MAT-C1 cells, respectively. Several experiments indicate that this component is at the cell surface in both sublines: 1) It is labeled by ^{125}I and lactoperoxidase

Figure 20. Polyacrylamide Gel Electrophoresis in SDS of [^{3}H]Glucosamine Labeled MAT-B1 Cells (A,D), Particulate Shed Fraction (B,E) and Soluble Shed Fraction (C,F). Gels A-C were stained with Coomassie blue and D-F are fluorograms showing the radioactive components. Note the predominance of ASGP-1 in all labeled fractions.

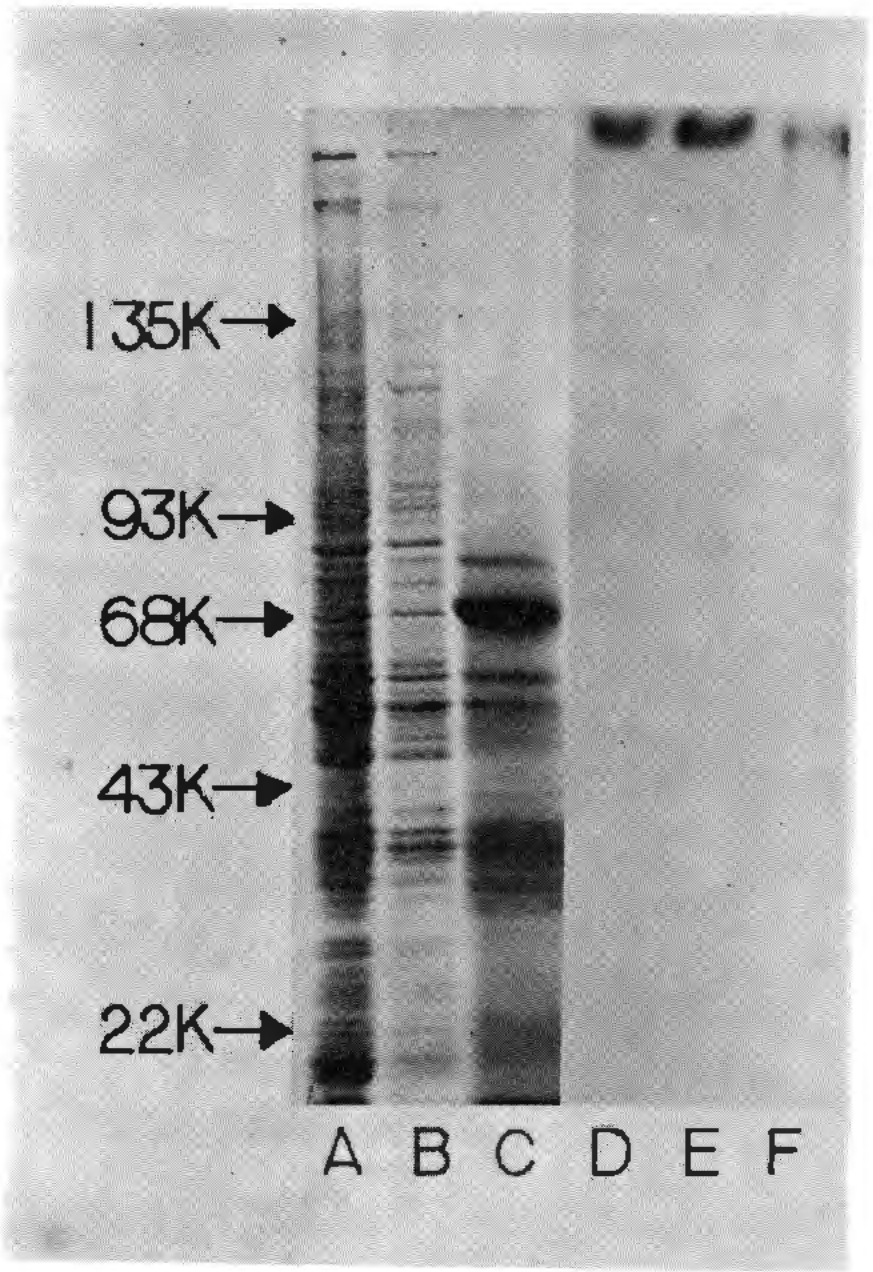
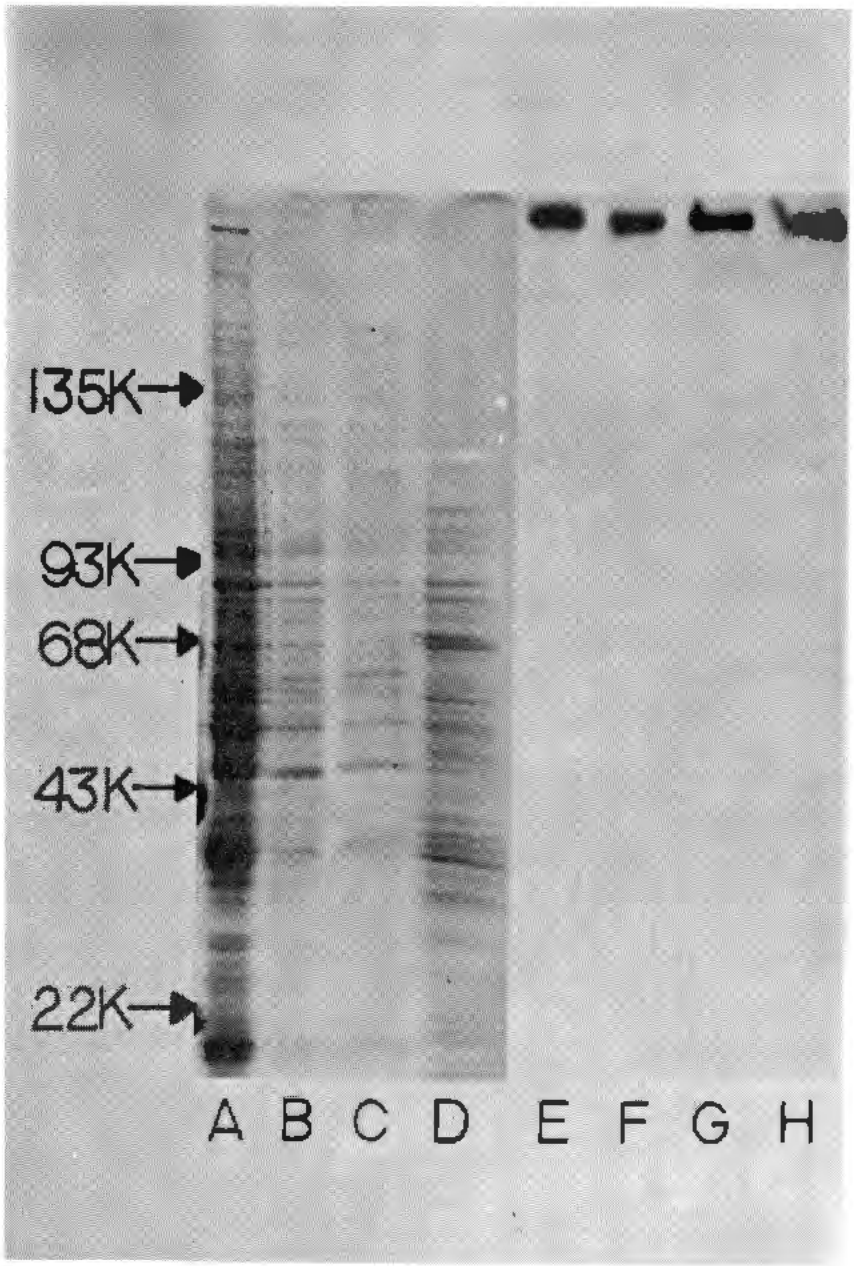

135K
93K
68K
43K
22K
A B C D E F

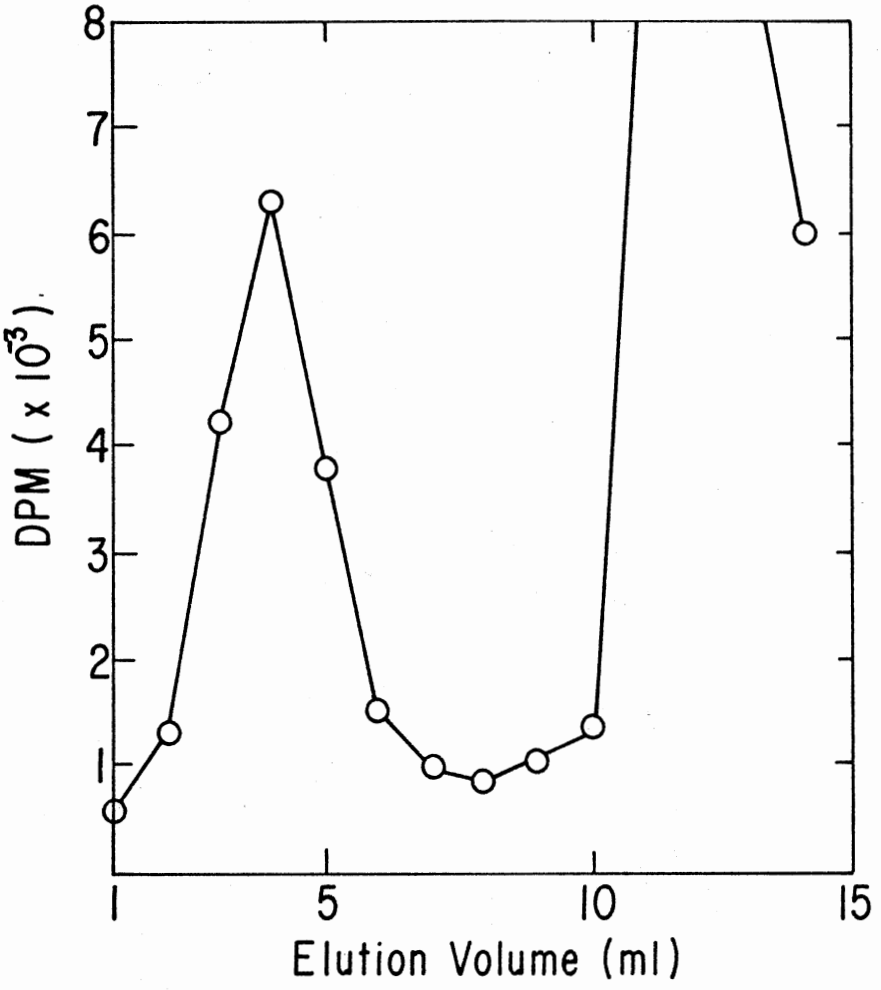
Figure 21. SDS Polyacrylamide Electrophoresis of [^{3}H]Glucosamine Labeled MAT-C1 Cells (A,E), Shed Microvilli Fraction (B,F), Shed Membrane Fragment and Vesicle Fraction (C,G) and Shed Soluble Material (D,H). Conditions are as in Figure 20.


135K
93K
68K
43K
22K
A B C D E F G H

(Sherblom et al., 1980c). 2) Glucosamine label can be released by trypsin or chymotrypsin from the cells (Buck, Sherblom and Carraway, 1979). When cells are treated with Pronase, most of the ASGP-1, assayed by SDS polyacrylamide gel electrophoresis and autoradiography, is degraded before the permeability barrier of the cells is broken (Huggins et al., 1980b). 3) ASGP-1 co-isolates with 5'-nucleotidase on putative plasma membrane fractions obtained from a microsome preparation or from a Zn^{++} stabilized membrane preparation (Sherblom, Buck and Carraway, 1980a).

When cells are incubated in vitro, released cell surface material can be observed by dark field microscopy or by fluorescence microscopy after staining with fluorescent Con A. MAT-C1 microvilli were identified by the unusual morphology of the branched microvilli (Huggins et al., 1980b). The presence of ASGP-1 in these shed fractions was ascertained in prelabeled cells by low speed centrifugation to remove the cells and quantitation of the radioactivity in the supernatant. SDS polyacrylamide gel electrophoresis of the supernatants confirmed the presence of ASGP-1. However, differential centrifugation did not adequately separate cells from the shed insoluble material. Therefore Percoll gradient centrifugation was used (Huggins et al., 1980b). Three fractions are obtained from a Percoll gradient for MAT-C1 cells; two are found for MAT-B1 cells. By microscopy these were identified as 1) microvilli, 2) membrane fragments or vesicles and 3) cells. The middle fraction is absent for MAT-B1 cells. Although the three fractions can be obtained in pure form from a carefully controlled gradient, the microvilli and fragment fractions tend to overlap somewhat. Figure 22 shows a typical Percoll gradient profile for shed material from MAT-C1 cells in which fractions 5 and 6 contain predominantly fragments and 2-4 contain predominately microvilli. For the results

Figure 22. Percoll Gradient Profile of MAT-C1 Cells (Major Peak) and Particulate Fraction (Smaller Peak) Shed *In Vitro*. Gradient was run as previously described (Huggins *et al.*, 1980b).


8
7
6
5
4
3
2
1
DPM (x 10⁻³)
1
5
10
15
Elution Volume (ml)

presented in this work we have considered the insoluble, shed fractions together for quantitative analysis. The soluble fraction of shed material was obtained by centrifugation at 100,000 x g for one hr. The shed, particulate material is quite heterogeneous in size (Howard et al., submitted for publication). Dark field microscopy indicates that microvilli predominate in the heavier fractions.

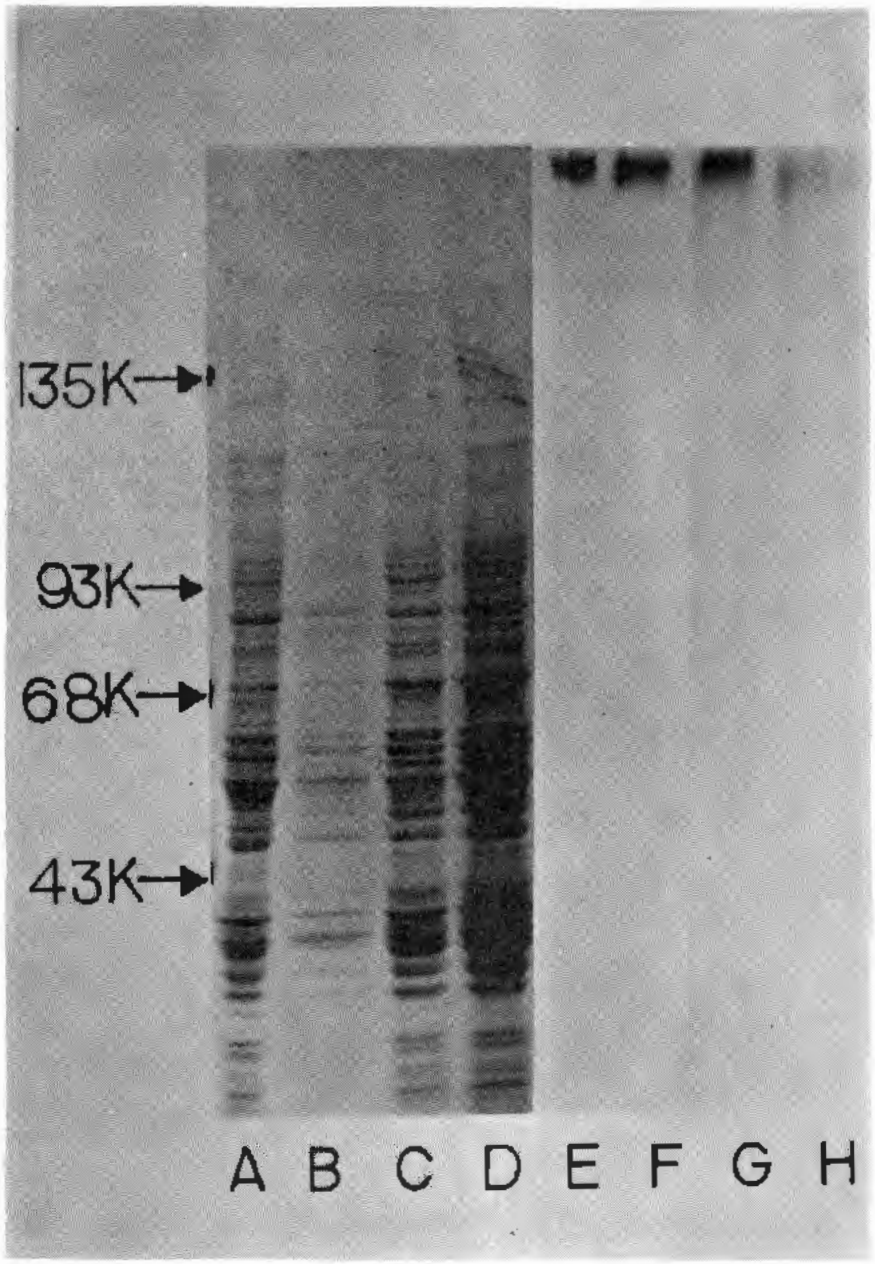
Examination of the labeled shed material by SDS polyacrylamide electrophoresis and fluorography showed ASGP-1 to be the only significant labeled component in the soluble fractions (Figures 20 and 21). Microvilli and the fragment (vesicle) fraction also contain predominately ASGP-1, although a small amount of a second labeled component (ASGP-2) was present but not readily discernible in the photographs of Figures 20 and 21.

To show that the soluble ASGP-1 could have been released from the cell surface rather than secreted from within the cell, labeled MAT-B1 cells were treated with trypsin, which cleaves cell surface ASGP-1 (Buck, Sherblom and Carraway, 1979). After incubation with trypsin inhibitor the cell suspension was washed to remove protease and incubated in medium for two hr. Released, soluble ASGP-1 was obtained after centrifugation. Electrophoresis and fluorography indicated that essentially all of the released ASGP-1 from the trypsin treated cells had been cleaved (Figure 23), indicating that it had come from the cell surface rather than from within the cell.

Kinetics of Shedding Processes

To investigate the release process, MAT-B1 and MAT-C1 cells were labeled in vivo, transferred to culture and incubated in the presence of

Figure 23. Release of Soluble ASGP-1 from [^{3}H]Glucosamine Labeled MAT-B1 Cells With and Without Trypsin Pretreatment. Electrophoresed whole cells are shown in (A), isolated microvilli in (B), soluble ASGP-1 released from MAT-B1 cells without trypsin pretreatment (C) and soluble ASGP-1 released after trypsin pretreatment (D). The corresponding fluorogram is shown in wells E-H in the same order.


135K
93K
68K
43K
A B C D E F G H

cold glucosamine. Aliquots were removed at intervals and fractionated to give cells, particulate shed material and soluble shed material. Release of both particulate and soluble material was linear for at least eight hours in both MAT-B1 and MAT-C1 (Figure 24). Since there was no lag in the release of soluble material, ASGP-1 appears to be released directly from the cells rather than requiring further degradation of released microvilli or membrane fragments, although a slow release of particulate material followed by rapid release of the soluble glycoprotein from its membrane would give the same result. The rates of shedding, based on the slopes of the lines, are quite similar for the two sublines for both soluble and particulate material.

Loss of label from the cells follows a first order relationship (Figure 25) with half-lives of 4.1 $\pm$ 0.5 da and 2.4 $\pm$ 0.2 da (3 experiments each) for MAT-B1 and MAT-C1 cells, respectively. Since the rate of turnover of label is greater than loss by shedding and is different between MAT-B1 and MAT-C1 cells, it is clear that turnover is more complex than shedding. Other processes must be involved to account for the turnover. Figure 26 shows that, using [^{3}H]leucine incorporation, MAT-B1 cells have a slower rate of synthesis of ASGP-1 than MAT-C1 cells although both cell lines exhibit similar levels of synthesis of total protein and actin.

Perturbation of the Shedding Processes by Effectors

Since ASGP-1 is the predominant component of the surface of 13762 ascites cells, the mechanism by which it is shed is of interest. Cellular ASGP-1 is membrane-bound, although it can be released from the membranes

Figure 24. Release of Soluble (▲, △) and Particulate (●, ○) Fractions from MAT-B1 (▲, ●) and MAT-C1 (△, ○) Cells Incubated In Vitro. Ascites cells were labeled with [^{3}H]Glucosamine.

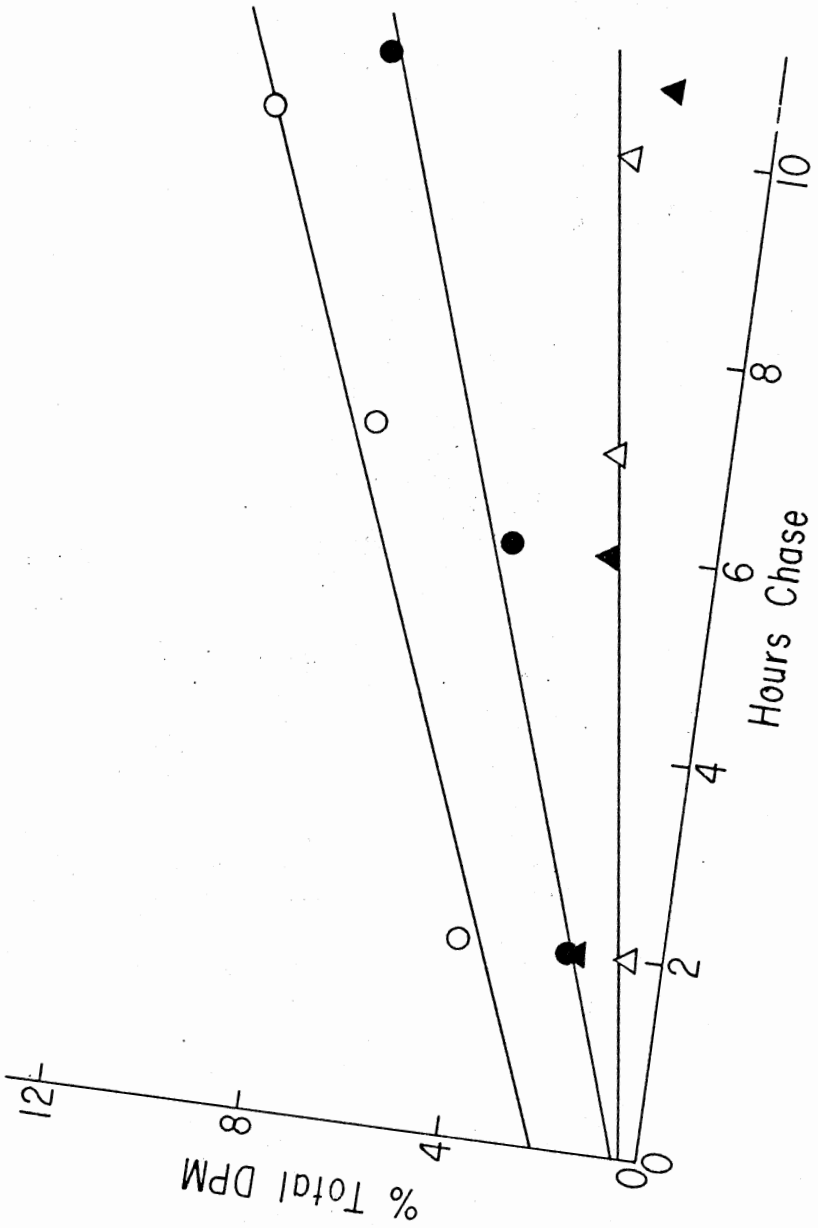

% Total DPM
0
4
8
12
Hours Chase
2
4
6
8
10

Figure 25. Loss of [^{3}H]Glucosamine Labeled Material from MAT-B1 (Δ) and MAT-C1 (○) Cells.

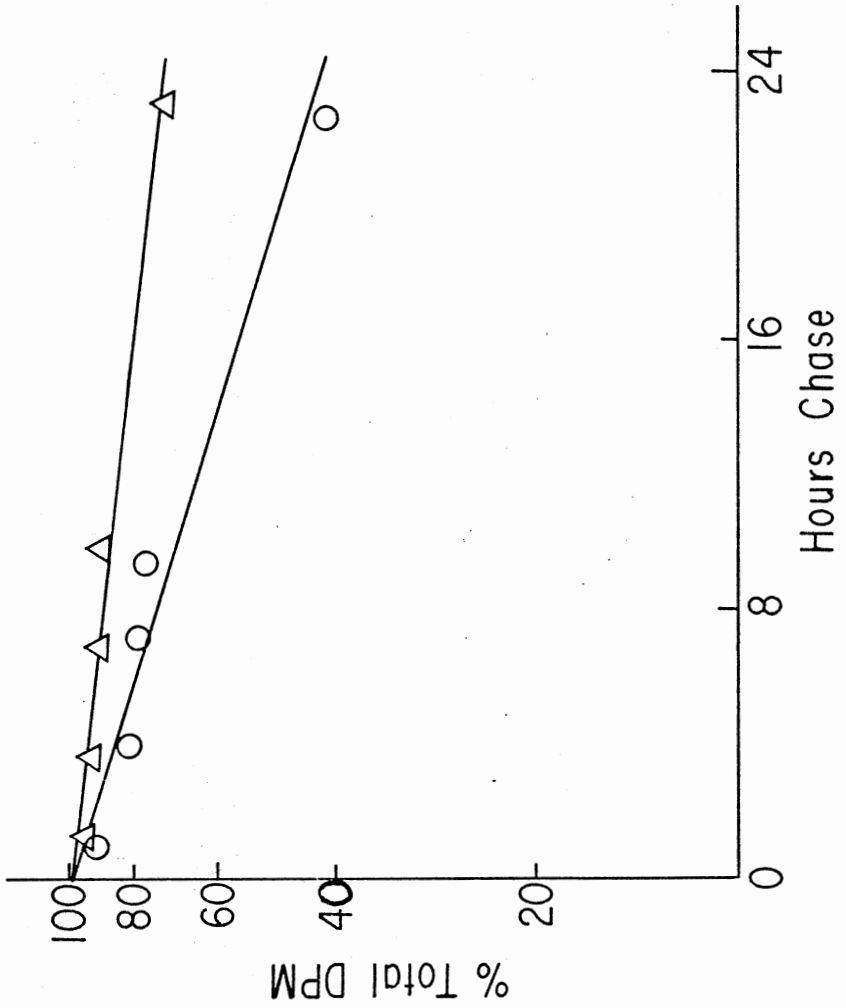
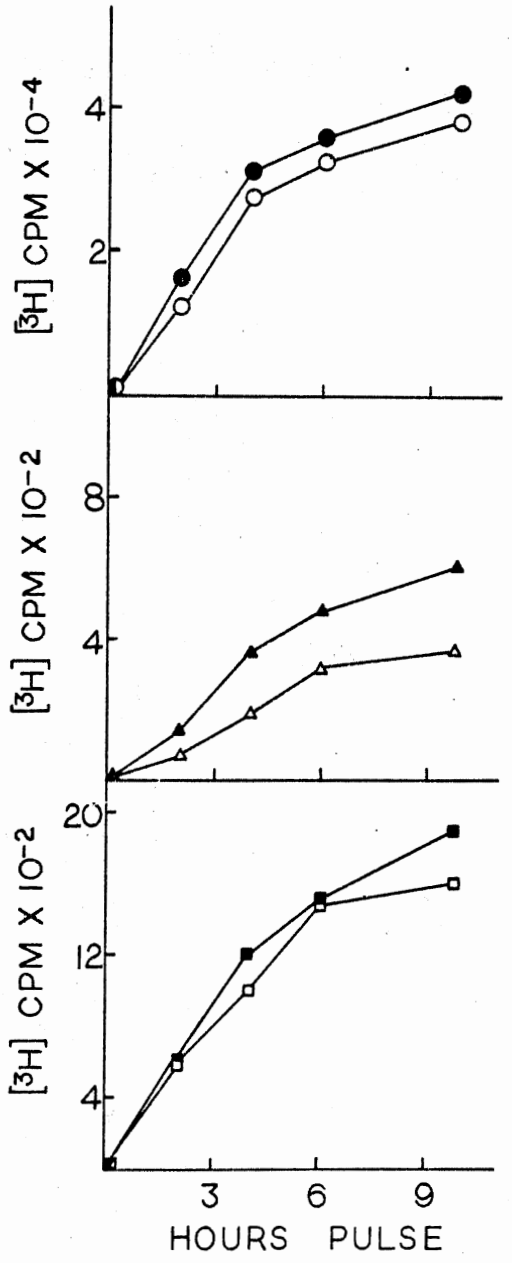

% Total DPM
100
80
60
40
20
0
8
16
24
Hours Chase

Figure 26. Incorporation of [^{3}H]Leucine into MAT-C1 (●, ▲, ■) and MAT-B1 (○, △, □) TCA-Precipitable Proteins (●, ○), Whole Cell ASGP-1 (▲, △) and Whole Cell Actin (■, □) in In Vitro Short-Term Culture with 6.7 μCi/ml and Cell Densities 6-7 x 10^{6} Cells/ml.


[3H] CPM X 10-4
4
2
[3H] CPM X 10-2
8
4
[3H] CPM X 10-2
20
12
4
3
6
9
HOURS PULSE

with guanidine hydrochloride (Sherblom, Buck and Carraway, 1980a) or nonionic detergent (Buck, Sherblom and Carraway, 1979). The fact that part of the shed ASGP-1 is soluble indicates that it must be altered in the shedding process. No change in apparent molecular size is observed by polyacrylamide gel electrophoresis. However, the negative charges and anomalous detergent binding characteristics of sialoglycoproteins make SDS polyacrylamide gel electrophoresis procedures unreliable for evaluating molecular sizes and size changes. Coworkers have compared the gel filtration in SDS profiles of ASGP-1 from the shed fractions and ASGP-1 purified from plasma membranes (Howard et al., submitted for publication; Sherblom, Buck and Carraway, 1980a). They have found ASGP-1 of the soluble fraction to be significantly smaller than purified ASGP-1, suggesting a cleavage mechanism of release.

As a further characterization of the mechanisms of the shedding processes, a number of effectors were tested for their ability to inhibit or accelerate release of soluble ASGP-1 or particulate surface material. Only Aprotinin and EDTA, of the effectors tested, were able to inhibit release of soluble ASGP-1 and each caused a 30-35% inhibition. None of the effectors gave a consistent, significant inhibition of release of the insoluble fraction. Shedding of both soluble and particulate fractions was enhanced by dibucaine (soluble 1.7-fold; particulate, 2.4-fold) and ionophore A 23187 in the presence of calcium (soluble, 2.7-fold; particulate, 3.5-fold). The effects on the release of soluble ASGP-1 provide further support for a proteolytic mechanism, possibly involving a cation-dependent protease. The effects of dibucaine and ionophore suggest a role for Ca^{++}.

Shed Materials in Ascites Fluid of Animals Bearing MAT-B1 and MAT-C1 Tumors

To determine whether similar shedding processes occur in vivo, ascites cells and fluid were removed from animals injected with [^{3}H]glucosamine. Immediate fractionation of the material on Percoll gradients gave results similar to those found for in vitro shedding with a broad peak at the density of the insoluble material. High speed centrifugation of labeled ascites fluid gave a soluble fraction which by SDS polyacrylamide gel electrophoresis (not shown) contained ASGP-1 as the only detectable labeled component. ASGP-1 was also the predominant labeled component of the particulate fraction isolated by Percoll gradient centrifugation (Howard et al., submitted for publication).

Consistent with their results from in vitro shedding, coworkers have shown that soluble ASGP-1 from MAT-C1 cells is smaller than the corresponding purified ASGP-1 using gel filtration in SDS and CsCl density gradient centrifugation. ASGP-1 from the particulate fraction isolated from ascites fluid is approximately the same size as purified ASGP-1 from MAT-C1 cells. Similar results were obtained for MAT-B1 cells (Howard et al., submitted for publication).

Dark-field microscopy showed only membrane fragments and vesicles from the ascites fluid of both MAT-B1 and MAT-C1 cells, indicating that any intact microvilli shed from the cell surfaces must be degraded in the animal. Scanning electron microscopy of cell-free, glutaraldehyde-fixed samples of ascites fluid showed stringy aggregates of cell surface material (not shown). No significant differences were observed between samples from MAT-B1 and MAT-C1 tumors.

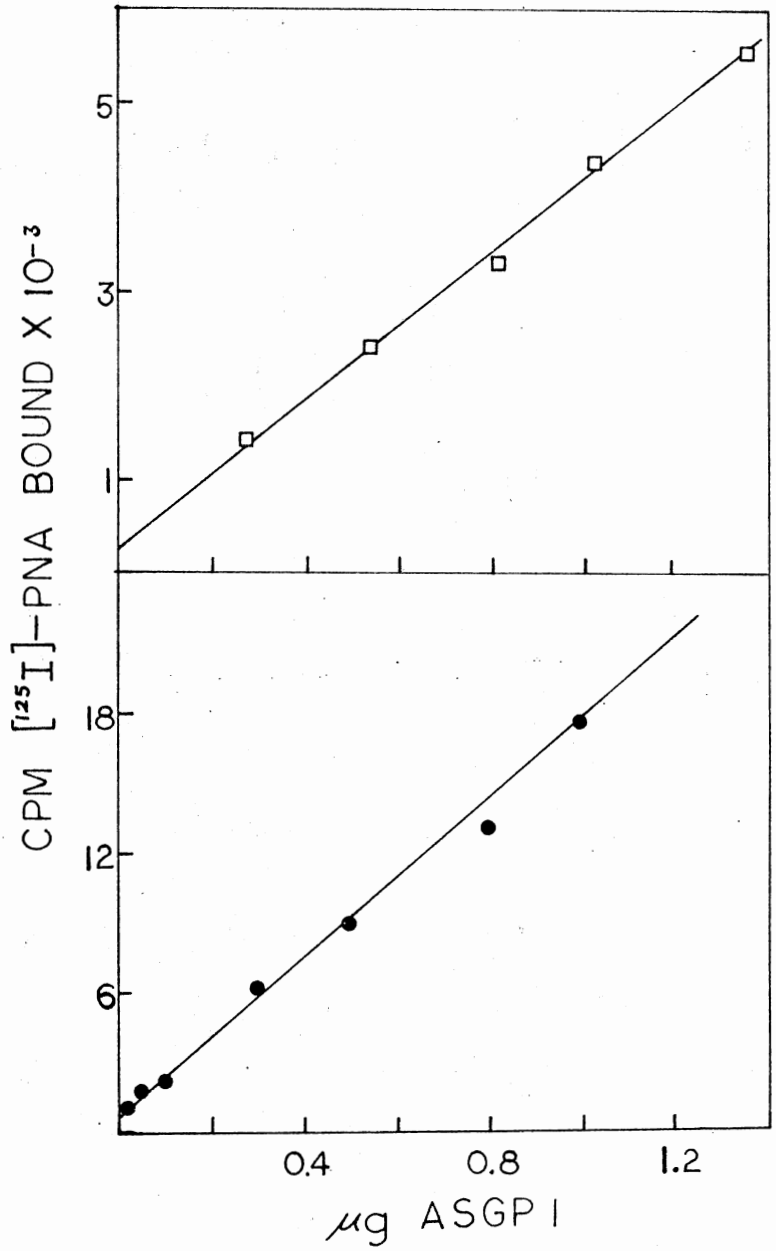
The amounts of particulate shed material and soluble shed ASGP-1 in ascites fluid were quantitated by protein analysis and the radiochemical lectin assay shown in Figure 27, respectively. The particulate fraction represented 0.8% and 0.6% of the total ascites fluid protein for MAT-B1 and MAT-C1 cells, respectively. Soluble ASGP-1 protein was 0.12% of the total ascites protein for MAT-B1 cells and 0.29% for MAT-C1 cells. Thus there is about 2.5 times as much soluble ASGP-1 in MAT-C1 ascites fluid as in MAT-B1, but about equal amounts of particulate fraction in the two sublines.

Discussion

Since shedding of cell surface glycoproteins such as ASGP-1 has been postulated to be important to transplantability across strain and species barriers (Cooper et al., 1974; Miller and Cooper, 1978b; Miller, Cooper and Brown, 1978a), we have investigated the mechanisms, rates and components of the shedding process. MAT-B1 and MAT-C1 cells shed cell surface material in both particulate and soluble forms. Moreover, shed components can be observed in the medium of cells incubated in vitro or in ascites fluid removed directly from the animal. Although morphologically identifiable cell surface structures (e.g. microvilli) can be observed in the medium after shedding in vitro, no discrete structural elements are observed in ascites fluid. Thus it appears that shed structural elements break down in the ascites fluid. The structures and amounts of particulate surface products are quite similar for MAT-B1 and MAT-C1 cells in vivo, suggesting that shedding of particulate material is unlikely to account for biological differences between the sublines.

Since gel filtration in SDS has shown that the soluble shed form of

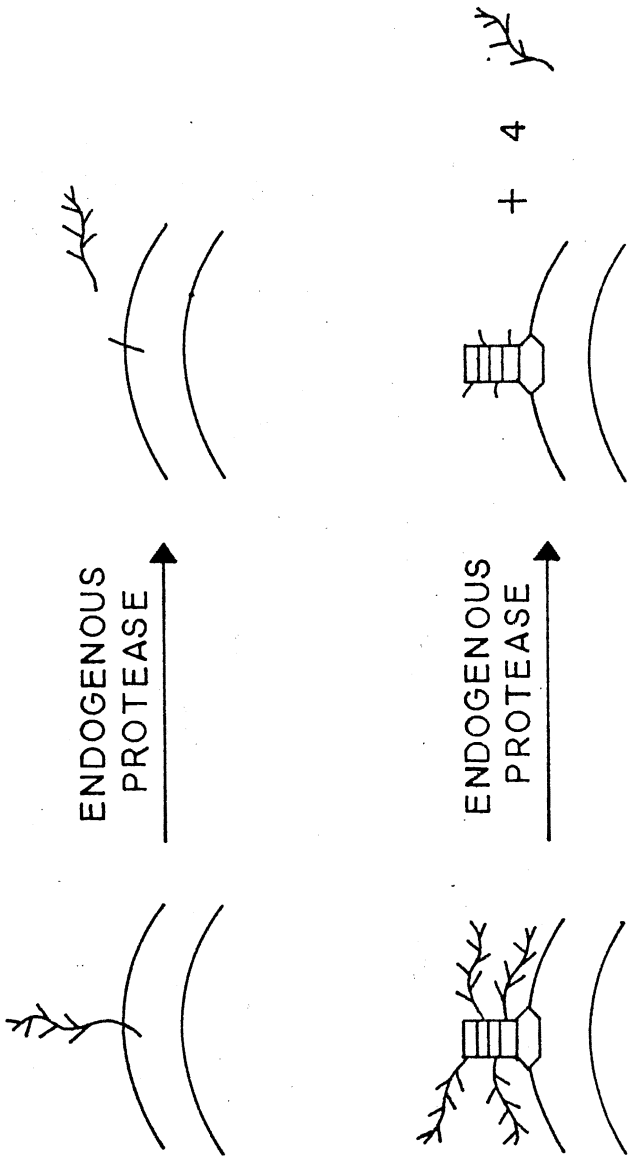
Figure 27. [^{125}I]PNA Assay for MAT-C1 and MAT-B1 (□, ●) ASGP-1. Samples were electrophoresed and stained with [^{125}I]PNA. ASGP-1 bands were then cut out and counted for radioactivity using a gamma counter. Protein values are based on a Lowry determination (Lowry et al., 1951).


CPM [125I]-PNA BOUND X 10-3
5
3
1
18
12
6
0.4
0.8
1.2
μg ASGP I

ASGP-1 is smaller than purified ASGP-1, which is approximately the same size as ASGP-1 released directly from cells by SDS treatment (Howard et al., submitted for publication; Sherblom, Buck and Carraway, 1980a), we suggest that release of ASGP-1 occurs by proteolytic cleavage. Removal of a portion of the polypeptide chain would not necessarily affect migration on SDS polyacrylamide gel electrophoresis where the detergent binding and charge of the sialic acid are important factors, but would probably alter the Stokes radius of the glycoprotein, which determines gel filtration behavior. The mode of binding of ASGP-1 to the membrane is unknown. One possibility is that a hydrophobic segment of ASGP-1 binds to the membrane bilayer. Release could occur by proteolytic cleavage of the polypeptide at a point between the membrane attachment and the bulk of the carbohydrate. This model, depicted in Figure 28, is similar to those postulated for cytochrome b_5 (Strittmatter, Rogers and Spatz, 1972) and erythrocyte glycophorin. However, since the sialoglycoprotein of MAT-C1 cells appears to be present in fibrillar structures on the cell surface (Sherblom et al., 1980c), it is questionable whether it interacts directly with the membrane. An alternate possibility is that ASGP-1 exists in polymerized form on the cell surface, interacting with the membrane via a coupling molecule (Figure 28), possibly ASGP-2 (Sherblom, unpublished observations). This mechanism of binding could more readily explain the release of ASGP-1 from membranes by the hydrophilic protein denaturants (urea, guanidine hydrochloride), and by nonionic detergents.

The question of the importance of shedding to the survival of the tumor cells is still unsettled. The rates of shedding of both particulate and soluble labeled components are quite similar for the two sublines in vitro, but the MAT-C1 subline has a 2.5-fold greater amount of soluble

Figure 28. Alternate Models for the Release of Soluble ASGP-1 from Ascites Cell Surfaces. ASGP-1, the branched structure, is shown either (top view) extending into the bilayer or associated with a coupling molecule (bottom). In either case soluble ASGP-1 is released by proteolytic cleavage.


ENDOGENOUS
PROTEASE
ENDOGENOUS
PROTEASE
+ 4

ASGP-1 in the ascites fluid than does MAT-B1. This result indicates that the relative rates of shedding of soluble material must be different in vivo or, more likely, that other processes are important in determining the amount of soluble ASGP-1 in ascites fluid. Regardless, these quantitative differences in ascites fluid glycoprotein do not appear sufficient to account for transplantation differences between the sublines. Thus our results do not provide positive evidence that shedding of sialoglycoproteins is a major factor for the 13762 tumor in permitting transplantation across histocompatibility barriers, as suggested for the TA3 mammary adenocarcinoma (Cooper , Codington and Brown, 1974; Miller and Cooper, 1978b; Miller, Cooper and Brown, 1978a), unless the shedding processes differ significantly for the sublines in the foreign host animal. We feel that it is more likely that other processes instead of or in addition to shedding are involved in escaping transplantation rejection. Further studies of this and similar systems are necessary to try to define these factors.

CHAPTER V

SUMMARY

Glycoproteins of a cultured form (13762 MR) of the 13762 rat mammary adenocarcinoma and its variants have been studied by analyses for peanut agglutinin (PNA) receptors, [^{3}H]glucosamine labeling, lactoperoxidase labeling and CsCl density gradient centrifugation. The 13762 MR cells, derived from 13762 MAT-B ascites cells, do not contain detectable ASGP-1, the predominant cell surface sialoglycoprotein of the ascites forms of the 13762 tumor. Transplantation and continued passages as ascites cells or MR cells or clonal lines derived from MR results in expression of ASGP-1, even though it is absent in early passages of the tumor. When these ascites cells are transferred to culture, ASGP-1 is again lost. No ASGP-1 is found in solid tumors derived from subcutaneous transplantation of the 13762 tumor cells, although clonal selection of populations with and without ASGP-1 cannot be unequivocally ruled out.

Two 13762 MR variant ascites cell lines, MAT-MR2-S and MAT-cMR6-S have been established through transplantation of the 13762 MR cells and a 13762 MR clone into F344 female rats. Both ascites lines have been partially characterized and differ from each other and from the previously established 13762 mammary adenocarcinoma ascites lines, including the MAT-B1 and MAT-C1 cells.

Because the shedding of cell surface components has been invoked as

a factor in the survival of tumor cells in a foreign host, the shedding processes in the xenotransplantable MAT-C1 and nonxenotransplantable MAT-B1 rat mammary adenocarcinoma sublines have been investigated. Using cells labeled *in vivo* with [^{3}H]glucosamine, it was shown that both sublines shed particulate and soluble cell surface material containing one predominant labeled component, the sialoglycoprotein ASGP-1. The rates of shedding of both particulate and soluble label are very similar for the two sublines, but the turnover of label in the cells was 80% greater for MAT-C1 cells ($t_{1/2}$ 2.4 da) than for MAT-B1 cells ($t_{1/2}$ 4.1 da). Based on [^{3}H]leucine incorporation, MAT-B1 ASGP-1 has a lower rate of synthesis than MAT-c1 ASGP-1. The release of the soluble form of ASGP-1 is decreased by the protease inhibitor Aprotinin or the addition of EDTA. Since coworkers have shown that ASGP-1 is sensitive to proteolytic cleavage (Buck, 1978) and that soluble ASGP-1 isolated either from ascites fluid or after *in vitro* incubation of ascites cells is smaller than ASGP-1 isolated from plasma membrane vesicles (Howard *et al.*, submitted for publication), the accumulated evidence suggests that soluble ASGP-1 arises from proteolytic cleavage. The MAT-C1 ascites fluid contained 2.5-fold more soluble ASGP-1 per mg protein than did MAT-B1. The amounts of shed particulate material in ascites fluid were similar for the two sublines, and no substantial morphological differences were observed for these particulate fractions by scanning electron microscopy. The results of these studies indicate that it is unlikely that shedding of prominent cell surface components contributes significantly to differences in ability of these tumors to transplant across species barriers.

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